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Detection of nonpregnant cows and potential embryo losses by color Doppler ultrasound and interferon-stimulated gene expression in grazing dairy cows

L. V. Madoz,[1,2]* S. N. Lorenti,[3]* R. Rearte,[1,2] L. Quintero-Rodriguez,[1,2]† A. L. Migliorisi,[1] M. Jaureguiberry,[1,2] C. Gabler, M. Drillich,[5] and R. L. de la Sota[1,2]‡

[1]Instituto de Investigaciones en Reproducción Animal (INIRA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, BA, Argentina B1900AVW
[2]CONICET, CABA, Argentina C1033AAJ
[3]Private practice, Brandsen, BA, Argentina B1980ABH
[5]Institute for Veterinary Biochemistry, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany 14163
[5]University Clinic for Ruminants, Clinical Unit for Herd Health Management in Ruminants, Department for Farm Animals and Veterinary Public Health, University of Veterinary Medicine, Vienna, Austria 1210

ABSTRACT

Many studies have been conducted to estimate pregnancy losses between 19 and 34 d after artificial insemination (AI) in dairy cows managed under confinement-based systems, but few studies have examined embryo mortality during this interval in dairy cows managed under gazing systems. The objectives of this prospective cohort study were (1) to assess the diagnostic value of the corpus luteum (CL) blood perfusion (BP) evaluation by Doppler ultrasound (US) to detect nonpregnant cows at 19 to 20 d post-AI, and (2) to assess the rate of potential embryo mortality between 19 to 34 d post-AI. The CL-BP of all cows included in the study (n = 131) was examined on farm by power and color mode of Doppler US and later using an image processing software by a second evaluator. The endometrium thickness and echotexture were evaluated by B-mode US at the same visit to assess if the nonpregnancy diagnosis could be improved at 19 to 20 d post-AI by this additional diagnostic tool. Blood samples were obtained at 19 to 20 d post-AI for progesterone (P4) measurement by chemiluminescence and to determine the mRNA expression of ISG by real-time PCR. Pregnancy diagnosis based on embryo visualization was performed at 33 to 34 d post-AI by US B-mode. In parallel interpretation, *ISG15* and *MX2* mRNA expression in leukocytes [sensitivity (Se), 100%] were regarded as suitable biomarkers for early pregnancy and were selected for molecular characterization of pregnancy at 19 to 20 d post-AI. At 19 to 20 d post-AI, 61.1% of the cows had positive CL-BP by Doppler US (Se, 98.0%), 62.7% had ISG mRNA expression in leukocytes over the cutoff point (Se, 95.7%), and 50.8% were positive, based on the combination of ISG mRNA expression, CL-BP by Doppler US, and P4 concentration (Se, 100%), and were considered as possible pregnant. At 33 to 34 d, the pregnancy rate was 37.4% diagnosed by the B-mode US. Based on the expression of the selected biomarkers in cows with active CL, we found that 28.1% of the cows could have potentially lost their pregnancy between 19 and 34 d post-AI. The Doppler US color mode showed similar accuracy and a higher negative predictive value than the genes selected as biomarkers. The additional B-mode ultrasound evaluation of the uterine stratum vasculare and the endometrium thickness improved the diagnostic accuracy. Therefore, assessing the CL-BP by Doppler US allowed early detection of nonpregnant cows at 19 to 20 d post-AI. The combination of early CL-BP by Doppler US (d 19 to 20) with early embryo detection by B-mode US (d 33–34) could be used to facilitate earlier rebreeding of dairy cows.

Key words: Doppler ultrasound, interferon-stimulated genes, luteal blood perfusion, diagnosis

INTRODUCTION

Reproductive efficiency in dairy cows is a function of conception rate and pregnancy loss (Fricke, 2002). Therefore, early identification of nonpregnant cows and early pregnancy losses improve the reproductive efficiency of dairy herds because they reduce the interval between consecutive inseminations and allow cows that lost a pregnancy to be re-inseminated earlier (Fricke, 2002).

Under commercial conditions, diagnosis of nonpregnant cows can be made between 26 and 33 d postinsemination quickly and accurately using a real-time B-mode ultrasonogram (**US**) 5 MHz transducer [>97% sensitivity (**Se**), >87% specificity (**Sp**); Pieterse et al.,

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*These authors contributed equally to this work.
†Current address: Universidad de Santander, Facultad de Ciencias Agrícolas y Veterinarias, Bucaramanga, Colombia 680002.
‡Corresponding author: dairydoc82@gmail.com

1990; Romano et al., 2007]; 5 to 10 MHz transducer (>92% Se, >83% Sp; Szenci et al., 1998; Nation et al., 2003; Silva et al., 2007; Karen et al., 2015). More recently, color flow Doppler US has become available to monitor blood flow in the corpus luteum (**CL**) around 20 d postinsemination to diagnose nonpregnant cows accurately [99% Se, 98.5% negative predicted value (**NPV**); Siqueira et al., 2013; Dubuc et al., 2020].

In dairy cows, pregnancy losses greatly affect the farm's reproductive and economic performance. Most of the losses occur during the first 60 d of pregnancy at embryonic or early fetal stages (Wiltbank et al., 2016). Fertilization failure represents only a minor proportion of the loss because the fertilization rate in dairy cows is typically high (>80%) and just slightly lower than in beef cattle (Bridges et al., 2013; Lonergan et al., 2016). On d 7 after AI, only 50 to 60% of developed embryos are still viable. Then, embryo losses average 30 and 12% between the pregnancy intervals of 8 to 27 d and 28 to 60 d, respectively (Wiltbank et al., 2016).

The detection of embryo losses in cattle can be assessed using different diagnostic tools. Transrectal US is the diagnostic tool most used in veterinary practice that allows the absence of the fetal heartbeat, detached membranes, floating structures (including embryo remnants), and reduction in fetal size from 28 d post-AI to be detected (Silke et al., 2002; Quintero Rodríguez et al., 2019). Other diagnostic tools that can be used for early detection of embryo mortality are measuring progesterone (**P4**) concentration in milk or blood at 20 to 24 d post-AI and assessing blood perfusion (**BP**) in the reproductive tract using Doppler US (Ealy and Seekford, 2019). There is a positive correlation between CL-BP and circulating P4 concentrations in cyclic and pregnant cows (Herzog et al., 2010; Rocha et al., 2019). Furthermore, the evaluation of CL-BP using Doppler US was used in cows to evaluate recipients for embryo transfer, estimate fertility before AI, and predict luteolysis (Berger et al., 2017; Varughese et al., 2017; Pugliesi et al., 2019).

Concerning the early molecular diagnosis of pregnancy in cows, the detection of IFN-τ stimulated genes (**ISG**) was applied in different studies as a reliable predictor [selected genes >85% of the area under the curve (**AUC**), receiver operator characteristic (**ROC**) analysis; Wijma et al., 2016; Yoshino et al., 2018]. Between 14 and 21 d of pregnancy, the trophoblast produces IFN-τ in large quantities, which is the signal for maternal recognition of pregnancy in ruminants and an essential regulator of uterine histotroph embryo elongation (Wiltbank et al., 2016). Blood leukocytes respond to IFN-τ by expressing ISG, which can be detected in peripheral blood between 18 and 22 d of pregnancy in heifers and cows (Gifford et al., 2007; Green et al., 2010).

Although there were studies that evaluated embryo losses in dairy cattle under confined conditions (Han et al., 2006; Monteiro et al., 2014; Wiltbank et al., 2016), there has not been any objective estimation of the percentage of pregnancy loss between 19 and 34 d post-AI in lactating Holstein cows reared under grazing conditions. The first objective of this study was to assess the diagnostic value of the CL-BP evaluation by Doppler US to detect nonpregnant cows at 19 to 20 d post-AI. In addition, we assess if uterine echotexture by B-mode US evaluation could add valuable information to improve the detection of nonpregnant cows at 19 to 20 d post-A. The second objective of this study was to assess the potential embryo mortality (**EM**) rate between 19 and 34 d post-AI, based on the difference between pregnancies identified on d 19 to 20 (combination of detecting ISG expression in peripheral blood leukocytes and identification of active CL using Doppler US and P4 measurement) and d 33 to 34 (embryo visualization using the B-mode US). The hypotheses to test were that the CL color Doppler US will allow detecting nonpregnant cows at 19 to 20 d post-AI under grazing conditions and that the estimation of potential EM between 19 and 34 d post-AI in grazing dairy cows is similar to those previously reported in confined systems.

MATERIALS AND METHODS

Herd Management

A prospective cohort study design was performed on a commercial dairy farm located in Brandsen, Buenos Aires province, Argentina (35°24′ S, 58°06′ W) with 400 milking Holstein dairy cows. All cows were under a grazing system supplemented with corn silage and concentrate. The accumulated milk yield per cow ranged from 6,000 to 12,000 kg per 305 d of lactation. Calving occurred all year-round, and the voluntary waiting period was set at 45 DIM. After the voluntary waiting period, cyclic cows with normal vaginal discharge (without pus) were selected and estrus synchronized. All cows received an intravaginal insert containing 1.2 g of P_4 (Diprogest 1200, Zoovet) and 2.0 mg of estradiol benzoate (Zoovet) at the beginning of the protocol. On the d 7 of the protocol, the P_4 insert was removed, and cows received 0.150 mg of $PGF_{2\alpha}$ (D-Cloprostenol, Ciclar Zoovet), 2 mg of estradiol cypionate (Zoovet), and 450 IU of eCG (Novormon 5000, Syntex). The AI was performed 55 to 60 h after the P_4 insert was removed.

Clinical and Ultrasound Examinations

Animal use in this study was approved by the Graduate School and the Laboratory Animal Care and Use Committees of the Faculty of Veterinary Sciences at the National University of La Plata (IACUC Code #:92–1-19P).

A total of 131 healthy cows on 19 to 20 d post-AI and with a CL, but no cows with cystic structures were enrolled. All CL were examined by power and color mode of Doppler US (ESAOTE MyLab OneVET) equipped with a linear 2.2 to 12 MHz transrectal transducer. The settings for power mode (frequency 6.6 MHz, total gain 55%, pulse repetition frequency 750 Hz, the frame rate of 23 frames/s, and minimum detectable velocity of 0.06 m/s) and color Doppler mode (frequency 6.6 MHz, the total gain of 55% and pulse repetition frequency of 750 Hz) were fixed and used in all examinations to minimize variations among cows. The same investigator conducted all ultrasound evaluations.

The evaluation considered the amount of colored area within the luteal tissue as an indicator of CL functionality (Lüttgenau and Bollwein, 2014; Kaya et al., 2017). The most colored view of the largest diameter for each CL was selected. Each quadrant was classified on farm according to the apparent percentage of CL-BP (0–4 scale; 0: area with 0% vascularization, 1: 1–25%, 2: 26–50%, 3: 51–75%, and 4: 76–100%). Two video clip records containing the entire cross-section of the CL in power and color Doppler modes were stored for later image analysis. The clips were blind analyzed by a second evaluator independently. As in the on-farm CL-BP visual evaluation, the CL was divided into 4 equal quadrants. The percentage of colored areas in each quadrant was determined using image processing software ImageJ 1.42q (National Institutes of Health; Díaz et al., 2019). Three images of each CL were examined to calculate the colored area. The most vascularized picture of the 3 images was selected and used for the final statistical analysis. The classification scale was the same used on farm. The CL-BP was considered negative when had the 4 quarters scored as ≤ 1 (scale 0–4) or when the software evaluation indicated $<25\%$ of CL-BP (Pugliesi et al., 2019).

During the same visit on 19 to 20 d post-AI, an additional examination using B-mode ultrasound (ESAOTE MyLab OneVET) was performed to assess the uterine echotexture. The proportional rate of thickness between the endometrium (**EN**) and myometrium (**MY**) at the bifurcation of uterine horns (EN:MY) was determined and classified as 1:1, 2:1, and 3:1. The presence (yes or no) of the uterine lumen and stratum vasculare were also evaluated.

Pregnancy was diagnosed at 33 to 34 d post-AI by US B-mode (Mindray DP 30 Vet, frequency 8.0 MHz, total gain 110, and frame rate of 28 frames/s) based on visual identification of the embryonic vesicle and the heartbeat (Figure 1).

Cows diagnosed by Doppler US with a positive CL-BP at 19 to 20 d that were nonpregnant on 33 to 34 d by the B-mode US were called POS-NONPREG. Cows diagnosed by Doppler US with a negative CL-BP at 19 to 20 d and diagnosed as pregnant on 33 to 34 d were called NEG-PREG. Cows that were diagnosed positive in both US examinations were called POS-PREG. Finally, cows diagnosed with a negative CL at 19 to 20 d and nonpregnant at 33 to 34 d were called NEG-NONPREG.

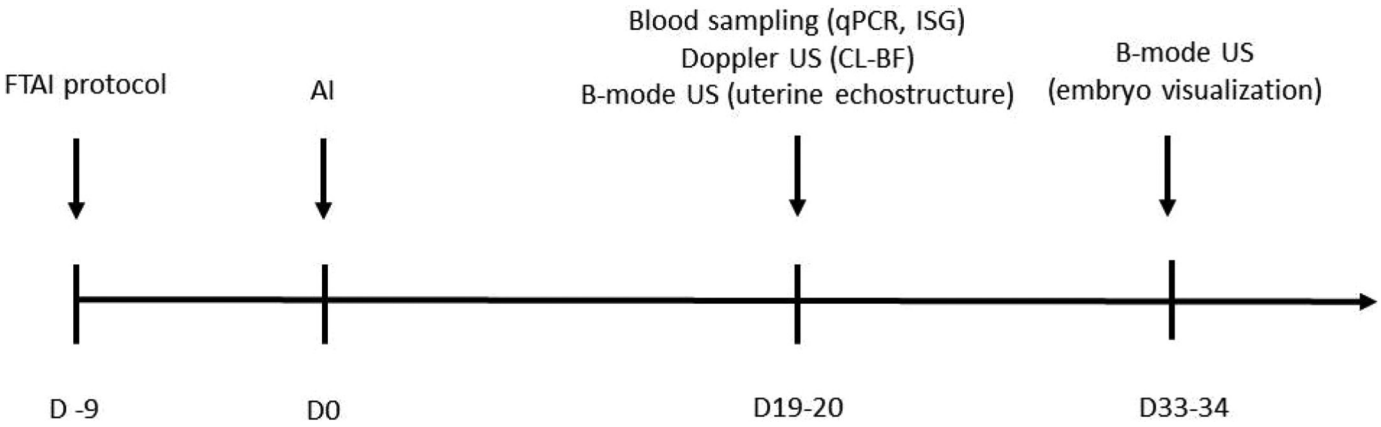

Figure 1. Timeline showing the study design and diagnostic techniques used in this study. FTAI = fixed-time AI; qPCR = real-time PCR; ISG = interferon-stimulated genes; CL-BP = corpus luteum blood perfusion; US = ultrasound.

Table 1. Selected gene transcripts, primer sequences, and annealing temperatures (Tm) were used for quantitative PCR and its normalization with resulting amplicon length

Gene	Gene sequence of nucleotide[1]	Reference or accession no.	Product size (bp)	Temperature (°C)
SDHA	For 5′-GGGAGGACTTCAAGGAGAGG-3′ Rev 5′-CTCCTCAGTAGGAGCGGATG-3′	Gärtner et al., 2016	219	60
SUZ12	For 5′-TTCGTTGGACAGGAGAGACC-3′ Rev 5′-GTGCACCAAGGGCAATGTAG-3′	Peter et al., 2015	286	60
ACTB	For 5′-CGGTGCCCATCTATGAGG-3′ Rev 5′-GATGGTGATGACCTGCCC-3′	Gärtner et al., 2016	266	58
INFAR1	For 5′-CAACTTTTCAAGCTCAGTGG-3′ Rev 5′-CTCTTCAGACCAAAAAGAGG-3′	NM_174552.2	200	55
INFAR2	For 5′-ACGAGAATCAGAGTCATCAG-3′ Rev 5′-TCCACACTTTCTTCTTCCTG-3′	NM_174553.2	245	55
ISG15	For 5′-CGCCCAGAAGATCAATGTGC-3′ Rev 5′-GCTTCAGCTGGACCTCATAG-3′	NM_174366.1	200	60
RTP4	For 5′-CAGCCCATCCAAGTAGCAAG-3′ Rev 5′-CTGAAGCTGTGTCCCTTGTG-3′	NM_001075961.1	213	60
OAS1	For 5′-CAGCTGGAAGCCTGTCAAAG-3′ Rev 5′-CTTGGATGAGCCGGACATAG-3′	NM_001040606.1	214	60
MX1	For 5′-AAGACAAGGTCGTGGACGTG-3′ Rev 5′-TTCCCTTCCTCCAGAAGGTC-3′	NM_173940.2	181	60
MX2	For 5′-GCATTGACCTCATCGACTCC-3′ Rev 5′-CCCGCTTTGTCAGTTTCAGC-3′	NM_173941.2	189	60

[1]For = forward; Rev = reverse.

Collection and Processing of Blood Samples

Blood samples were collected from the coccygeal vessels (9 mL/cow) at 19 to 20 d post-AI using a 10-mL syringe. Blood was immediately transferred to EDTA-containing tubes (K3 tubes, DVS Argentina) and placed on ice until processing. Within 5 h after collection, tubes were centrifuged at 1,200 × *g* for 12 min at 4°C. The plasma obtained was stored at −20°C until P_4 measurement. Then buffy coat fractions were collected and processed to obtain a peripheral blood leukocytes pellet as described (Gifford et al., 2007). To each leukocyte pellet, 0.5 mL Trizol (Invitrogen) was added and was gently pipetted up and down. The samples were stored at −80°C until RNA extraction.

Extraction of Total RNA and Reverse Transcription

Only 59 samples were available to be processed and used for further mRNA expression analysis (the remainder of the samples were discarded due to human error in the leukocytes pellet recovery step). The total RNA was extracted from the stored lysed leukocytes by the Trizol method following the manufacturer's instructions. Total RNA was quantified by spectrophotometry at a wavelength of 260 nm (Nanodrop, ND-1000, Peqlab Biotechnologie). To validate RNA's integrity, samples were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) following the manufacturer's protocol (RIN 9.2–9.9).

All samples were DNase I treated (Fermentas) to remove genomic DNA. Then, 1,000 ng of RNA per sample was used to generate single-stranded cDNA by using 2.5 m*M* random hexamer primers, 0.66 m*M* dNTP, 1× buffer reverse transcriptase, and 200 U of reverse transcriptase MMLV (Fermentas) in a total volume of 60 μL as described in detail (Odau et al., 2006). Samples without reverse transcriptase were used as negative controls to confirm the absence of genomic DNA or contaminations.

Real-time PCR

Real-time PCR assays for selected ISG were carried out as described previously in detail (Odau et al., 2006) using specific primers designed by Primer 3 Software (Untergasser et al., 2012), as indicated in Table 1. A total reaction volume of 10 μL per sample for real-time PCR contained 1 μL of cDNA, 0.2 μ*M* each primer (F and R), and 5 μL of 2× SensiMix SYBR Low-ROX (Bioline). Amplification was carried out using the Rotor-Gene 3000 (Corbett Research). A 3-step amplification followed a denaturation step of 95°C for 10 min in 45 cycles, denaturation at 95°C for 15 s, annealing for 20 s (temperatures are indicated in Table 1), and extension at 72°C for 30 s. A subsequent melting curve program (50–99°C) with continuous fluorescence measurement was carried out to confirm specific amplification, followed by a final cooling step to 40°C. A dilution series with known concentrations of the purified specific PCR products were amplified simultaneously to generate a standard curve. Rotor-Gene 6.1 software (Corbett Research) was used to calculate specific mRNA contents compared with the standard curves.

The mRNA expression of the genes of interest was normalized with the corresponding normalization factor calculated with the mRNA expression of reference genes suppressor of zeste 12 homolog (*SUZ12*), β-actin (*ACTB*), and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*; Table 1) by geNorm (Vandesompele et al., 2002). Normalized data were used for statistical analysis.

Determination of P4 Concentrations

Progesterone concentration in plasma was measured by chemiluminescence (Immunoanalyzer Elecsys and Cobas e, Roche; Jaureguiberry et al., 2020; Madoz et al., 2021). The assay's precision was determined by including control samples with low (1 ng/mL) and high (7 ng/mL) P4 concentrations by duplicates at the beginning and the end of the assay. Intra-assay coefficients of variation were 2.0% for the low P4 control (0.3 ng/mL) and 4.5% for the high P4 control (4.87 ng/mL) samples. Cows that had plasma P4 concentration ≥1.0 ng/mL were classified as having functional CL, and those with P4 concentrations <1.0 ng/mL were classified as having nonfunctional CL. Active CL was considered when CL-BP was classified as positive to Doppler US and functional to P4 measurement.

A total sample size of 50 cows was calculated to detect a pregnancy loss of 35 percentage units from a 70% pregnancy rate at 19 to 20 d post-AI ($\alpha = 0.05$ and $\beta = 0.20$; G*Power 3.1, University of Dusseldorf; Faul et al., 2007). A total sample size of 126 cows was calculated to detect a kappa coefficient of 0.80 with a 2-tailed test null value of 0.60 and 80% of power (Sim and Wright, 2005) with a 70% pregnancy rate at 19 to 20 d post-AI. The cow was considered the experimental unit.

Statistical Analysis

The agreement between the on-farm diagnosis by Doppler US (i.e., nonpregnant) and the CL-BP area by image processing software (ImageJ 1.42q) was assessed by estimating the kappa coefficient. The predictive capacity of diagnosis of nonpregnancy at 19 to 20 d post-AI of the color mode Doppler US was assessed by estimating the Se, Sp, positive predictive value, and NPV using Proc FREQ (SAS 9.4).

The concordance among CL-BP Doppler diagnosis at 19–20 d, pregnancy status by the B-mode US at 33–34 d, and the uterine echostructure at 19–20 d (uterine lumen, stratum vasculare, thickness rate EM: MY) were assessed by fitting univariable logistic regressions models (Proc GLIMMIX, SAS 9.4) and Se, Sp, positive predictive value, and NPV were estimated by Proc FREQ (SAS 9.4).

The ISG mRNA expression was used as the reference method for pregnancy at 19 to 20 d post-AI (Green et al., 2010; Ribeiro et al., 2014; Kose et al., 2016; Yoshino et al., 2018) in cows with an active CL. The ROC curves analysis was used to determine critical cutoff values for the genes of interest that provided evidence of an embryo's presence at 19 to 20 d post-AI (Proc LOGISTIC, SAS 9.4). For this, only cows diagnosed as POS-PREG and with P4 concentration > 1.0 ng/mL and NEG-NONPREG were included in the analysis. The critical cutoff value was defined at the point on the ROC curve that had the greatest AUC. To improve predictive capacity, the genes that were selected as biomarkers of pregnancy (AUC >80%) were used in several combined ways (parallel and series tests; Dohoo et al., 2003) for pregnancy diagnosis (Proc FREQ, SAS 9.4). Once the ISG thresholds were determined, all POS-NONPREG cows were reclassified according to their mRNA expression values and CL activity as potential EM (cows positive to ISG and active CL) or Doppler false positive (cows negative to ISG or with a nonactive CL). Potential pregnancy loss was calculated as the number of cows classified as EM divided by the number of cows diagnosed pregnant at 33 to 34 d by embryo visualization plus EM cows.

The agreement between the CL-BP diagnosis by Doppler US, pregnancy diagnosis by B-mode US, mRNA expression values, and P4 concentration was assessed by estimating the kappa coefficients (Proc FREQ, SAS 9.4). Significance was set at $P < 0.05$, and a trend for significance was set at $P < 0.10$.

RESULTS

The nonpregnancy rate at 19 to 20 d of all cows included in this study diagnosed by Doppler US was 38.9% (51/131), whereas 62.6% (82/131) were diagnosed as nonpregnant at 33 to 34 d by the B-mode US based on lack of embryo visualization.

At 19 to 20 d post-AI, from the total of cows with mRNA expression analysis, 62.7% (37/59) had ISG expression levels above the threshold (parallel interpretation of ISG15/MX2). Of those cows, 55.9% (33/59) had P4 > 1.0 ng/mL, and 50.8% (30/59) were positive CL by Doppler US at 19 to 20 d post-AI. In addition, the diagnosis by the B-mode US detected as pregnant 38.9% (23/59) of those cows at 33 to 34 d.

The CL-BP area measurements performed at 19 to 20 d post-AI by image processing software had a greater agreement with color mode than with power mode (kappa = 0.70 vs. 0.42). The presence of considerable CL-BP was considered a positive sign of an active CL, whereas its absence or small amounts (4 quarters of the CL scored as ≤1, scale 0–4) denoted a lack of CL activ-

Table 2. Proportions of true positive, true negative, false positive, false negative, sensitivity, specificity, negative predictive value, positive predictive value, and accuracy for the corpus luteum (CL) blood perfusion and for the parallel combination[1] of *ISG15* and *MX2* (*ISG/MX2*; n = 59) at 19–20 d after AI[2]

Endpoint	CL Doppler (n = 131)	CL Doppler (n = 59)	*ISG15/MX2* (n = 59)
True positive,[3] n	48	22	22
True negative,[4] n	50	17	21
False positive,[5] n	32	19	15
False negative,[6] n	1	1	1
Sensitivity,[7] %	98.0 (48/49)	95.7 (22/23)	95.7 (22/23)
Specificity,[8] %	61.0 (50/82)	47.2 (17/36)	58.3 (21/36)
Negative predictive value,[9] %	98.2 (50/51)	94.4 (17/18)	95.5 (21/22)
Positive predictive value,[10] %	60.0 (48/80)	53.7 (22/41)	59.5 (22/37)
Accuracy,[11] %	74.8 (98/131)	66.1 (39/59)	72.8 (43/59)

[1]Positive when one of both genes are above the threshold of expression.

[2]Reference: ultrasound (US) B-mode at 33–34 d after AI).

[3]Positive to CL Doppler or *ISG15/MX2* at 19–20 d after AI and also positive to US B-mode at 33–34 d after AI.

[4]Negative to CL Doppler or *ISG15/MX2* at 19–20 d after AI and also negative to US B-mode at 33–34 d after AI.

[5]Positive to CL Doppler or *ISG15/MX2* at 19–20 d after AI and negative to US B-mode at 33–34 d after AI.

[6]Negative to CL Doppler or *ISG15/MX2* at 19–20 d after AI and also positive to US B-mode at 33–34 d after AI.

[7]Indicates the ability of the diagnostic test to identify pregnant cows correctly.

[8]Indicates the ability of the diagnostic test to identify nonpregnant cows correctly.

[9]Probability that cows with a negative diagnosis are truly nonpregnant.

[10]Probability that cows with a positive diagnosis are truly pregnant.

[11]Proportion of true results, either true positive or true negative results.

ity and so, considered as a sign of nonpregnancy. The color mode showed high accuracy to detect possible pregnancy on farm by simple visualization (74.8%) when using category ≥2 in any quadrant. Likewise, US Doppler, similarly to ISG diagnosis, showed high Se and NPV when B-mode US was the gold standard (Table 2). Moreover, Doppler US diagnosis of possible pregnancy showed a substantial agreement with P4 in plasma, a moderate agreement with the B-mode US at 33 to 34 d, and fair concordance with ISG for the series and parallel combination, respectively. All kappa values calculated to evaluate the relative quality of the diagnostic tests used in this study are shown in Table 3.

The presence of a thick endometrial layer (EM: MY score 3:1) at 19 to 20 d was correlated with nonpregnant diagnosis by Doppler and B-mode US at 33 to 34 d (*P*

Table 3. Kappa values calculated to evaluate the relative quality of the diagnostic tests used in this study for diagnosis of possible pregnancy: corpus luteum (CL) blood perfusion at 19–20 d after AI (CL Doppler; n = 131), ultrasonography (US) B-mode 33–34 d after AI (US B-mode; n = 131), plasma P4 concentration at 19–20 d after AI (P4; n = 59), interferon-stimulated genes: *ISG15* and *MX2* in series at 19–20 d after AI (*ISG15+MX2*; n = 59), interferon-stimulated genes: *ISG15* and *MX2* in parallel at 19–20 d after AI (*ISG15/MX2*; n = 59)

	Agreement between tests			
Comparison	Kappa[1]	ASE[2]	95% CI	Level of agreement
CL Doppler vs. US B-mode	0.52	0.06	0.39–0.64	Moderate
CL Doppler vs. P4	0.70	0.10	0.49–0.90	Substantial
CL Doppler vs. ISG (ISG15+MX2)	0.36	0.12	0.16–0.65	Fair
CL Doppler vs. ISs (ISG15/MX2)	0.40	0.12	0.15–0.64	Fair
US B-mode vs. P4	0.31	0.08	0.14–0.46	Fair
ISG (*ISG15+MX2*) vs. P4	0.28	0.09	0.10–0.45	Fair
ISG (*ISG15/MX2*) vs. P4	0.33	0.12	0.08–0.57	Fair
ISG (*ISG15+MX2*) vs. US B-mode	0.58	0.11	0.38–0.79	Moderate
ISG (*ISG15/MX2*) vs. US B-mode	0.49	0.10	0.29–0.68	Moderate
ISG (*ISG15+MX2*) vs. ISG (*ISG15/MX2*)	0.61	0.09	0.42–0.79	Substantial
ISG (*ISG15/MX2*) vs. ISG (*ISG15/MX2/OAS1*)	1	0	1–1	Perfect

[1]Kappa values between 0.2 and 0.4 indicate fair agreement, between 0.4 and 0.6 indicate moderate agreement, between 0.6 and 0.8 indicate substantial agreement, and > 0.8 indicate almost perfect agreement (Dohoo et al., 2003).

[2]ASE: alpha SE.

< 0.001). In addition, the visualization of the uterine stratum vasculare at 19 to 20 d (OR: 2.79; 95% CI: 1.31–5.93; $P < 0.01$) was also correlated with nonpregnant cows at B-mode diagnosis at 33–34 d (Table 4).

The *RTP4*, *MX1*, *INFAR1*, and *INFAR2* genes had low Se and AUC <80% for assessing pregnant and nonpregnant cows, so they were not used to evaluate EM detection. On the contrary, *ISG15*, *MX2*, and *OAS1* presented AUC >80% and high Se, so they were selected as biomarkers to detect EM. The gene *ISG15* gave the highest AUC value of the selected biomarkers, and the parallel interpretations of *ISG15*/*MX2* and *ISG15*/*MX2*/*OAS1* showed both 100% of Se and 70.5% of Sp to detect pregnant cows and were selected for this study (Figure 2). The cutoff criteria, Se, Sp, and AUC for the mRNA expression of the genes of interest and their parallel and series interpretation are based on cows considered POS-PREG and NEG-NONPREG (n = 39) and are shown in Table 5.

The POS-NONPREG cows were reclassified based on their P4 concentration and ISG mRNA expression values (parallel interpretation of *ISG15*/*MX2*). The 47.4% (9/19) of the POS-NONPREG cows had evidence of embryo presence (expression value above the cutoff for ISG15 or *MX2*) and presented P4 concentration > 1.0 ng/mL (Se, 100%), so they were classified as EM. The 5.2% (1/19) had low P4, and the 47.4% (9/19) presented low *ISG15*/*MX2* and were classified as Doppler false positive. Based on the cows classified with EM (n = 9) and those diagnosed pregnant at 33 to 34 d (n = 23), we estimate that 28.1% (9/23+9) of the cows could have presented a potential pregnancy loss.

All cows that had a nonfunctional CL (P4 < 1.0 ng/mL) at 19 to 20 d were diagnosed nonpregnant at 33 to 34 d by B-mode US (Se 100%, 13/13), whereas half of the cows with P4 concentration ≥1.0 ng/mL at 19 to 20 had an embryo detected at 33 to 34 d by B-mode US (Sp 50%, 23/46).

DISCUSSION

Color Doppler US has been described as a valuable tool to evaluate BP in large animal reproductive tracts (Pugliesi et al., 2019). In addition, the color mode had shown to be more efficient than the power mode to detect the blood flow in small vessels in the center of the CL (Bollwein et al., 2013). Because of that, and in concordance with previous studies, we choose the color Doppler US evaluation for this study (Lüttgenau and Bollwein, 2014; Dubuc et al., 2020). However, it is important to note that CL-BP assessment by image processing software is very time-consuming; therefore, only the on-farm visual evaluation can be considered appropriate in dairy practice.

Table 4. Percentage of uterine lumen (UL) presence, stratum vasculare (SV), and proportional rate of thickness between endometrium and myometrium (EN/MY) at 19–20 d according to the diagnosis by Doppler ultrasound (US) at 19–20 d and the B-mode US at 33–34 d post-AI (n = 131)

Item	Result	UL, %		SV, %		EN/MY, %		
		Yes	No	Yes	No	1/1	2/1	3/1
CL-BP Doppler US	Positive	48.7 (41/80)	51.2 (39/80)	62.5 (50/80)	37.5 (30/80)	5.0 (4/80)	92.5 (74/80)	2.5 (2/80)***
	Negative	53.0 (27/51)	47.0 (24/51)	70.6 (36/51)	29.4 (15/51)	7.8 (4/51)	56.9 (29/51)	35.3 (18/51)***
B-mode US	Pregnant	42.8 (21/49)	57.1 (28/49)	51.0 (25/49)	48.9 (24/49)	8.2 (4/49)	91.8 (45/49)	0.0 (0/49)***
	Nonpregnant	57.3 (47/82)	42.6 (35/82)	74.4 (61/82)**	25.6 (21/82)**	4.8 (4/82)	70.7 (58/82)	24.4 (20/82)***

**$P < 0.01$.
***$P < 0.001$.

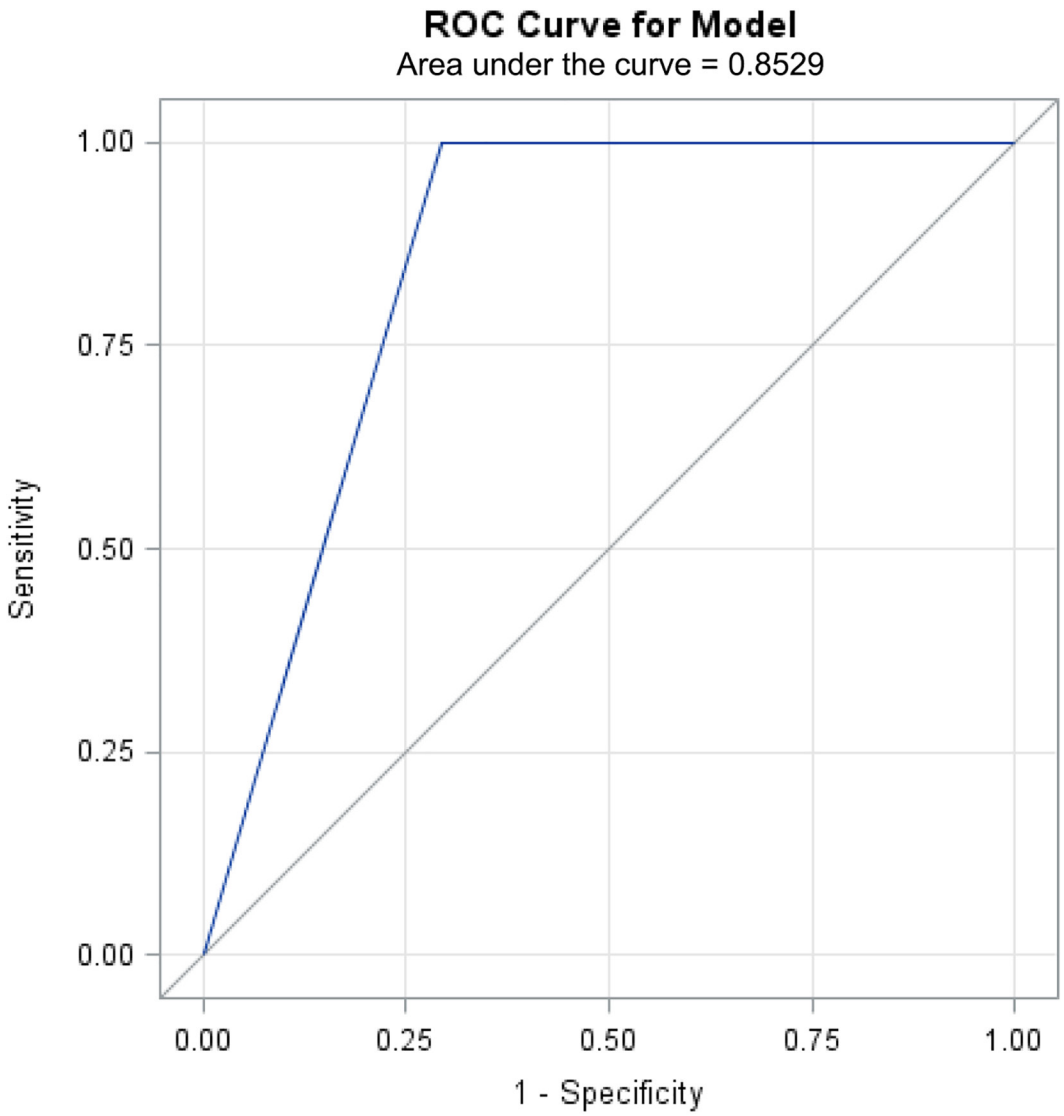

Figure 2. Receiver operating curve (ROC) of the model using the cutoff points for *ISG15* and *MX2* in parallel combination for the detection of pregnant cows at 19–20 d post-AI.

Our results showed high agreement with those reported by Siqueira et al. (2013) in Holstein-Gir crossbred that found Se 99%, Sp 53.7%, and NPV 98.5% for diagnosis by color Doppler US at 20 d using the diagnosis by the B-mode US at 30 d as reference (pregnancy rate 40.3%). The high NPV indicates that the assessment of the CL-BP seems to be a reliable diagnostic tool to detect open cows on farm and re-inseminate them earlier than would be possible by assessing return to estrus (Siqueira et al., 2013). However, when we compared the assessment of the CL-BP with the ISG expression, we detected that there was only a fair agreement, so the eligibility of ISG to serve as a reference method to detect nonpregnant cows at 19 to 20 d post-AI is limited. Reducing the calving to conception interval by an early and accurate nonpregnancy diagnosis may have significant economic importance. However, in agreement with previous studies we found that the CL-BP assessment presented false positive results and lowered accuracy as a limitation (Guimarães et al., 2015; Dalmaso de Melo et al., 2020). Incorrect pregnancy diagnosis may occur in estrous cycles with extended luteal phases with a delay in CL regression and early embryo losses (Giordano et al., 2012).

We found that a thicker endometrium and the visualization of the stratum vasculare at 19 to 20 d were related to nonpregnancy. That is in concordance with previous studies that found that the uterine wall thickness increases during the luteal regression with the diminishing of P4 and the increase of estradiol (Sugiura

Table 5. Cutoff value, sensitivity (Se),[1] specificity (Sp),[2] and area under the curve (AUC) with 95% CI obtained by receiver operating characteristic curve analysis for *ISG15*, *MX2*, and *OAS1* and their combinations to predict pregnancy at 19–20 d post-AI[3]

Gene	Combination[4]	Cutoff value	Se, %	Sp, %	AUC	95% CI
ISG15	No	0.22	0.95 (21/22)	0.82 (14/17)	0.89	0.78–0.99
MX2	No	3.70	0.86 (19/22)	0.82 (14/17)	0.84	0.72–0.96
OAS1	No	0.12	0.73 (16/22)	0.88 (15/17)	0.80	0.68–0.93
ISG15/MX2	Parallel	0.22–3.70	1.00 (22/22)	0.71 (12/17)	0.85	0.74–0.96
ISG15/OAS1	Parallel	0.22–0.12	0.95 (21/22)	0.76 (13/17)	0.86	0.75–0.97
MX2/OAS1	Parallel	3.70–0.12	0.86 (19/22)	0.82 (14/17)	0.84	0.72–0.96
ISG15+MX2	Series	0.22–3.70	0.81 (18/22)	0.94 (16/17)	0.88	0.79–0.98
ISG15+OAS1	Series	0.22–0.12	0.73 (16/22)	0.94 (16/17)	0.83	0.72–0.94
MX2+OAS1	Series	3.7–0.12	0.72 (16/22)	0.88 (15/17)	0.80	0.68–0.93
ISG15+MX2+OAS1	Series	0.22–3.70–0.12	0.63(14/22)	0.94 (16/17)	0.79	0.67–0.90
ISG15/MX2/OAS1	Parallel	0.22–3.70–0.12	1.00(22/22)	0.71 (12/17)	0.85	0.74–0.96

[1]Sensitivity (Se %): indicates the diagnostic test's ability to identify pregnant cows correctly. Se was calculated by receiver operating characteristic curve analysis using Positive-Pregnant cows (n = 22/39).

[2]Specificity (Sp %): indicates the diagnostic test's ability to identify nonpregnant cows correctly. Sp was calculated by receiver operating characteristic curve analysis using Negative-Nonpregnant cows (n = 17/39).

[3]Data are based only on animals considered Positive-Pregnant or Negative-Nonpregnant (n = 39).

[4]Parallel: a cow is considered pregnant when at least one of the results is positive (expression values above the cutoff point). Series: a cow is considered pregnant only when both results are positive (expression values above the cutoff point).

et al., 2018). Similarly, the visualization of the stratum vasculare is explained by the increase in uterine arteries' cross-section diameters and blood flow as an influence of the increasing estradiol concentrations. It is concordant with the positive correlation reported between the maximum uterine time-averaged flow velocity (using Doppler US) and the plasma estrogen levels between days −3 and −1 of the bovine estrous cycle (Bollwein et al., 2000; Abdelnaby et al., 2018). So, including evaluating the stratum vasculare and the thickness of the endometrium as an additional assessment may improve nonpregnant cow diagnosis at 19 to –20 d post-AI.

In this study, we reported that the estimation of potential pregnancy rate in lactating grazing dairy cows at 19 to 20 d post-AI was 61.1% (Doppler US) and 62.7% (ISG), 50.8% (ISG+ Doppler US + P4), and at 33 to 34 d was 37.4% (B-mode US). The apparent pregnancy loss for the 19 to 34 d period was 28.1%. The occurrence of embryo losses that we found is similar to other studies (25–40.5%), also using ISG as the reference method for early pregnancy detection during a similar pregnancy period. However, those studies presented methodological differences from our study, had a higher number of false negative results and were carried out under a confined system (Han et al., 2006; Monteiro et al., 2014; Wiltbank et al., 2016) or in beef cattle (Dalmaso de Melo et al., 2020). In addition, it is important to notice that in our study, all cows considered as a candidate for embryo loss had an active CL at d 19–20. As the gold standard for pregnancy diagnosis at 19–20 d for this study, we selected the series interpretation of the 3 diagnostic techniques (P4 + CL-BP by Doppler + ISG). It is the best estimation possible to obtain from the available diagnostic techniques. As far as we know, this is the first study reporting estimation of pregnancy losses in dairy cattle diagnosed by ISG under pastoral conditions; however, we are aware that this study represents only one farm. More studies with more farms and under different grazing conditions and seasons could contribute to understanding embryo mortality in grazing dairy cows.

It can be hypothesized that cows detected as EM exhibited interferon-tau secretion evidence at 19 to 20 d, but the amount and timing might have been insufficient to prevent luteolysis (Wijma et al., 2016). In addition, the presence of a nonviable embryo can also secrete IFN-τ and lead to an extension of the CL lifespan (Wiltbank et al., 2016). The measurement of plasma P4 concentration provides the best reliable information on luteal function (Lüttgenau and Bollwein, 2014). In agreement with a study carried out by Siqueira et al. (2013), we found that P4 concentration in plasma at 19 to 20 d post-AI allows detecting all pregnant cows (Se 100%) diagnosed at 33–34 d post-AI; however, there were also a considerable number of false positives. In addition, similar Sp was found between studies (50 vs. 51.7%). The accuracy of P4 for pregnancy diagnosis that we found was slightly less than the diagnosis of CL-BP by Doppler US performed on the same day (61 and 74.8%, respectively). Similar P4 accuracy in predicting pregnant cows (58%) was reported by Han et al. (2006).

The joint assessment of plasma P4 concentrations and the mRNA expression of ISG provides an excellent overview of the CL function and the presence of a viable embryo (Sheikh et al., 2018). Kose et al. (2016) reported that after a PGF2α administration in sheep, the embryo (based on ISG detection) could survive

longer than the CL. It could explain some positives to ISG mRNA expression that did not show high P4 concentration.

The ISG mRNA expression is similar between cyclic nonpregnant cows before insemination (Green et al., 2010) but then increases only in pregnant cows. Thus, these genes were mentioned as a good predictor of pregnancy. In our study, *ISG15*, *MX2*, and *OAS1* showed evidence of the presence of an embryo and were chosen as possible biomarkers. Several studies selected these genes for indirect evaluation of the existence of interferon-tau in the peripheral blood of cows and heifers (Gifford et al., 2007; Green et al., 2010; Shirasuna et al., 2012; Kizaki et al., 2013; Sheikh et al., 2018; Melo et al., 2020). However, the ROC curves derived from our and previous studies showed that false positive and false negative rates would be found in lactating cows regardless of the pre-defined cutoff (Green et al., 2010; Pugliesi et al., 2014; Yoshino et al., 2018). The lack of ISG accuracy for pregnancy diagnosis is probably the combination of different factors. For instance, the ISG mRNA expression varies between the parity categories, presenting heifers with higher mRNA expression levels than multiparous cows (Green et al., 2010). In addition, ISG mRNA expression could be stimulated in another physiological status not associated with pregnancy, such as virus exposures, increasing false positives diagnosis (Yoshino et al., 2018). Because of the chance of physiological variations in the ISG expression, selecting positives cows to at least one biomarker (parallel interpretation of ISG expression) will ensure that all possible pregnant cows will be detected; however, embryo mortality could be potentially overestimated. Although the lack of ISG accuracy for diagnosis, there are no better options for biomarkers to detect early pregnancy. Other pregnancy markers, such as pregnancy-associated glycoproteins, could be used but are indicated after d 24 (Pohler et al., 2016).

In cows with EM around the time the interferon-tau signal maintains the CL, ISG mRNA expression patterns should be more similar to pregnant than cyclic cows or cows with no embryo (Wijma et al., 2016). Our AUC values for the selected genes *ISG15* and *MX2* were similar to those reported by Yoshino et al. (2018) and Wijma et al. (2016), indicating that these genes have good Sp and Se and are suitable to study early embryo death. The *OAS1* mRNA abundance had the least AUC value of the 3 selected genes of interest, as in the Yoshino et al. (2018) study; nevertheless, the *OAS1* AUC value in our study was higher than 80% and therefore was selected as a possible biomarker. To improve the efficiency of pregnancy diagnosis, a combined assessment of 2 or more genes (series interpretation of ISG) can be used to increase the accuracy of the pregnancy diagnosis due to the reduction in false positive results (Dalmaso de Melo et al., 2020). We observed that the parallel combination of the 3 genes selected as biomarkers (*ISG15*/*MX2*/*OAS1*) or just 2 of them in parallel combination (*ISG15*/*MX2*) improves the efficiency of detecting pregnant cows and showed 100% of Se and 70.5% of Sp, allowing the detection of embryo losses. In addition, *ISG15*/*MX2* showed very high Se and NPV and resulted in fewer false negative cows than previous studies that estimated EM (Han et al., 2006; Monteiro et al., 2014; Wiltbank et al., 2016). This novel finding indicates that a parallel combination of *ISG15*/*MX2* improves Se and reduces false negatives, resulting in almost all negative cows for *ISG15*/*MX2* being truly nonpregnant. Pregnancy detection yielded 72.8% accuracy, similar to the accuracy reported by Han et al. (2006) and Pugliesi et al. (2014).

CONCLUSIONS

Doppler US was demonstrated to be a good option to quickly detect nonpregnant cows so they can be rebred earlier, minimizing days open. Nevertheless, we suggest evaluating the stratum vasculare and endometrium thickness as an additional assessment to improve the detection of nonpregnant cows at 19 to 20 d post-AI. In combination with pregnancy diagnosis by the B-mode US at 33 to 34 d could be a useful, practical application for resynchronization protocols in veterinary dairy practice.

The parallel interpretation of *ISG15* and *MX2* was the only combination of ISG that showed to be accurate and 100% Se identifying pregnant cows, allowing potential embryo losses detection. However, we propose to evaluate ISG mRNA expression, Doppler US, and P4 altogether in series interpretation to improve the estimation of embryo mortality between 19 and 20 d to 33 to 34 d. Based on that interpretation, we estimate 28.1% potential embryo loss between 19 and 34 d post-AI in Holstein cows on a supplemented grazing dairy system.

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