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Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination

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ABSTRACT

The objective was to determine the accuracy of a pregnancy test for predicting nonpregnant cattle based on the evaluation of corpus luteum (CL) blood flow at 20 d (CLBF-d20) after timed artificial insemination (TAI). Crossbred Holstein-Gir dairy heifers (n = 209) and lactating cows (n = 317) were synchronized for TAI using the following protocol: intravaginal implant (1.0 g of progesterone) and 2 mg of estradiol benzoate i.m. on d −10, implant removal and 0.526 mg of sodium cloprostenol i.m. on d −2, 1 mg of estradiol benzoate i.m. on d −1, and TAI on d 0. On d 20, animals underwent grayscale ultrasonography (US) to locate the CL and color flow Doppler to evaluate CLBF-d20 using a portable ultrasound equipped with a 7.5-MHz rectal transducer. Based only on a visual, subjective CLBF evaluation, the animals were classified as pregnant or not pregnant. On d 30 to 35, blinded from results of the previous diagnosis, the same operator performed a final pregnancy diagnosis using US to visualize the fetal heartbeat (gold standard; US-d30). A second evaluator also analyzed the CLBF-d20 in the same animals by watching 7-s recorded videos. Blood samples were collected from a subset of 171 females to determine, by RIA, plasma progesterone (P_4) concentrations, which indicate CL function. The final pregnancy outcome (US-d30) was retrospectively compared with the CLBF-d20 diagnoses and then classified either as correct or incorrect. The number of true positive, true negative, false positive, and false negative decisions were inserted into a 2 × 2 decision matrix. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the CLBF-d20 test were calculated using specific equations. Binomial variables (pregnancy rate and proportions) were analyzed using Fisher's exact test for the effect of parity and to compare between evaluators and tests (CLBF-d20 vs. plasma P_4). The kappa values were calculated to quantify the agreement between CLBF-d20 and the gold standard (US-d30) and between evaluators. The performance parameters of CLBF-d20 test were as follows: sensitivity = 99.0%, specificity = 53.7%, positive predictive value = 65.1%, negative predictive value = 98.5%, and accuracy = 74.8%. False negatives represented only 0.4% of the exams. No differences existed in these parameters between evaluators (no. 1 vs. no. 2) and tests (CLBF-d20 vs. plasma P_4). Moreover, a high level of agreement was observed between evaluators (0.91). In conclusion, visual evaluation of CLBF-d20 represents a quick, reliable, and consistent diagnostic test that enables the early detection of nonpregnant cattle.
Key words: corpus luteum, blood flow, luteolysis, pregnancy

INTRODUCTION

Pregnancy diagnosis has long been a routine activity in the management of cattle reproduction (Cowie, 1948; cited by Fricke and Lamb, 2005). Its primary purpose is to detect, as early as possible, animals that have failed to conceive, determine the cause of pregnancy failure, and to determine whether to rebreed (Fricke and Lamb, 2005) or cull such animals. The early diagnosis and rapid rebreeding of nonpregnant animals reduces interinsemination intervals (Stevenson, 2005) and is undoubtedly part of the strategy used to improve reproductive performance (Fricke, 2002). Thus, it may be especially advantageous if protocols for timed AI (**TAI**) are used (Fricke et al., 2003).

There is a long history of attempts to diagnose pregnancy in cattle at an early stage (Ghannam and Sorensen, 1967; Ludwick and Rader, 1968). Nevertheless, practical and reliable methods to generate a definitive diagnosis using ultrasonography at early stages of pregnancy (<25 d) remain of limited accuracy (**ACC**; based

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on predictive values) or may even be unavailable currently. Although previous reports have demonstrated that it is possible to successfully diagnose pregnancy as early as <20 d after breeding or AI using conventional grayscale ultrasonography (**US**; Kastelic et al., 1988, 1989, 1991; Pieterse et al., 1990), the reliabilities and accuracies of scans at early stages (d 21 to 25) are quite low (Pieterse et al., 1990; Quintela et al., 2012) most likely due to the inability to clearly visualize the embryo, which is commonly found close to the uterine wall during that stage of pregnancy (Quintela et al., 2012). Additionally, the possibility of confusing intrauterine estrus mucus with pregnancy remains (Pierson and Ginther, 1984; Kastelic and Ginther, 1989; Pieterse et al., 1990; Kastelic et al., 1991; Quintela et al., 2012). Consequently, pregnancy diagnosis by scanning the uterus to visualize the embryonic vesicle is of limited ACC, shows a relatively high incidence of misdiagnosis, and is not typically adopted in practice before 26 to 28 d of gestation (Pieterse et al., 1990; Romano et al., 2006; Quintela et al., 2012). The incorporation of new technologies in addition to the grayscale B-mode US, such as Doppler ultrasound, enables a more detailed assessment of the uterus, ovarian follicles, and corpora lutea. Color-flow mode (**CFM**) permits visualization of blood flow within tissues and structures based on the principles of the Doppler effect (Singh et al., 2003; Ginther, 2007; Matsui and Miyamoto, 2009) and indirectly enables inferences to be made on the functional status of the tissue (Herzog et al., 2010).

The establishment and maintenance of pregnancy in cattle is dependent on the presence of a functional, active corpus luteum (**CL**) and the production of a sufficient level of progesterone ($\mathbf{P_4}$; Mann and Lamming, 1999; Lucy, 2001; Parr et al., 2012). During luteolysis, the loss of luteal function (P_4 secretion) is associated with a progressive decrease in blood flow in response to $PGF_{2\alpha}$ (Niswender et al., 2000; Schams and Berisha, 2004). Morphological changes caused by luteal regression, however, only become evident later (Niswender et al., 1994; Siqueira et al., 2009). Thus, the usefulness of conventional B-mode US in evaluating CL function during the time of luteolysis is limited by the temporal asynchrony between functional and morphological regression (Kastelic et al., 1990; Assey et al., 1993; Siqueira et al., 2009). The use of color Doppler ultrasound can overcome these limitations because it enables the real-time assessment of CL blood flow (**CLBF**), which reflects the functionality of the gland indirectly, particularly by the end of the estrous cycle (Miyamoto et al., 2006; Herzog et al., 2010).

Interestingly, although it has been suggested that color Doppler flow imaging could be useful for a more accurate early diagnosis of pregnancy in cattle (Quintela et al., 2012), particularly if performed at 19 to 21 d after AI (Matsui and Miyamoto, 2009), results on the use of CLBF evaluations to determine pregnancy status are controversial. Recent studies reported that the evaluation of CLBF results alone was insufficient for the early diagnosis of pregnancy in bovine embryo transfer recipients due to low specificity (**Sp**) and sensitivity (**Se**; Utt et al., 2009) and was not a reliable diagnostic method for pregnancy in lactating dairy cows due to a high variation between animals (Herzog et al., 2011). However, this apparent inconsistency of CLBF may be due to differences in Doppler ultrasound settings, in the criteria used for data analysis, or in the time points used for CLBF evaluations relative to AI. A study of the performance of a CLBF-based pregnancy diagnosis test in a large TAI program, in which a great number of cows are inseminated and checked for pregnancy at a later time point may be an optimal strategy by which to address these questions on the potential for the use of color Doppler imaging in the routine reproductive management on dairy farms.

Thus, the objective of this study was to determine the ACC of a pregnancy diagnostic test, based only on the visual evaluation of CLBF using color Doppler flow imaging, on predicting nonpregnant animals at 20 d after TAI. We hypothesized that a single, subjective CLBF evaluation using color Doppler imaging after the expected luteolysis (d 20; **CLBF-d20**) would accurately detect the majority of animals that failed to conceive, thus enabling their early resynchronization.

MATERIALS AND METHODS

Animals, Experimental Design, and Estrus Synchronization

Holstein-Gir crossbred nulliparous heifers (20 to 22 mo and >330 kg of BW; n = 209) and multiparous lactating dairy cows (>3 yr old; n = 317), which were primarily Holstein (blood share of 77.0 ± 18.1%), with a mean BCS of 2.75 ± 0.56 (range: 2.0 to 4.0; scale 1 to 5; Edmonson et al., 1989) were enrolled in this study and subjected to 5 rounds of TAI. Lactating cows were >45 d DIM and their lactation records indicated an average production of 4,125 ± 641 kg of milk over a 305-d period, which was estimated using the highest milk yield during one of the previous lactations from each cow in the study. Cattle were maintained using a mixed land-based production system at the Embrapa Dairy Cattle Research Center located in Coronel Pacheco, Minas Gerais, Brazil. Animals were maintained on a pasture overnight and fed maize silage and grain ration as supplements during the day with ad libitum access to water, salt, and mineral mixture.

Both heifers and cows were synchronized for TAI using estradiol benzoate (**EB**; Sincrodiol; Ourofino Agronegócio, Ribeirão Preto, Brazil), P_4 intravaginal implants (1.0 g of P_4; Sincrogest; Ourofino Agronegócio), and a prostaglandin $F_{2\alpha}$ analog (sodium cloprostenol, Sincrocio; Ourofino Agronegócio). The ovulation synchronization protocol was as follows: 2 mg of EB i.m. and P_4 implant insertion on d −10, sodium cloprostenol (0.526 mg i.m.) and P_4 implant removal on d −2, a second administration of EB (1 mg i.m.) on d −1, and TAI on d 0 (52 to 54 h after implant withdrawal). Doses of commercial semen from bulls of proven fertility were used for AI, which was performed by a single technician. After TAI, animals returned to the farm's regular management until ultrasonographic examinations on d 20. All of the procedures using research animals were conducted in accordance with the Brazilian Ethics, Bioethics, and Animal Care Committee (CEBEA) guidelines and were approved by the Embrapa Ethics in the Use of Animals Committee (Protocol CEUA-CNPGL 02/2011).

B-mode and Doppler Ultrasound

Ultrasonography was performed on d 20 (d 0 = TAI) by a single operator. Day 20 was chosen arbitrarily for the evaluations based on the expected time of luteolysis in cattle. We decided that an earlier evaluation (d 17–18) would be of limited use because previous studies have reported a transient increase in blood flow surrounding the CL during the initiation of luteolysis (Miyamoto et al., 2005; Ginther et al., 2007) and the length of the luteal phase in nonpregnant cattle shows considerable variation (14 to 18 d; Forde et al., 2011). Later evaluations (after d 21) would also be confounded by the appearance of new corpus hemorrhagicum structures formed after possible ovulation, subsequent to a return to estrus in those animals that may have failed to conceive.

Ovaries of all of the animals enrolled in the study were scanned for CL identification using a B-mode portable ultrasound machine equipped with a 7.5-MHz linear-array, rectal transducer and color-flow Doppler mapping mode (MyLab30 Vet Gold; Esaote SpA, Genoa, Italy), which was used to visualize blood flow in the CL. During the CFM, the transducer frequency was set to 6.6 MHz, which was the maximum allowed frequency on the machine. The ultrasound imaging settings (B-mode frequency: 7.5 MHz; total gain: 70%; CFM frequency: 6.6 MHz; pulse repetition frequency: 1 KHz; and focus position: 1) were standardized and remained constant for all of the exams. After CL localization by conventional B-mode US, the CFM was activated and the operator (evaluator no. 1; L. G. B. Siqueira) visually evaluated the blood flow over the entire CL structure, which was referred to as the CLBF and was used as a pregnancy diagnostic test on d 20 (CLBF-d20). This subjective evaluation took into consideration the amount of colored pixels within the luteal tissue, which was considered to be an indicator of CL functionality or regression (Table 1). In addition to the real-time visual evaluation, a 7-s cine-loop (real-time clips in cine mode) was recorded over the entire CL (i.e., from one side to the other). The 7-s cine-loop video clips were recorded onto the internal hard drive of the ultrasound machine for future evaluations by evaluator no. 2 (J. H. M. Viana), who was not present during the d-20 examinations and who was blinded to all of the determinations of evaluator no. 1.

During both B-mode and Doppler ultrasonographic examinations, the uterus was not scanned. The transducer was positioned directly to scan the right and left ovaries. This criterion was defined in advance to make the Doppler evaluations rapid and straightforward, without the need for the detection of intrauterine fluid. Lastly, neither evaluator received any information on the possible return to estrus of the females enrolled in the study.

Prediction of Pregnancy Based on CLBF

The magnitude of CLBF was evaluated based on the detection of the Doppler signal in the CL. The presence of considerable CLBF was considered to be a positive

Table 1. Subjective criteria used by both evaluators as standards for determining whether the classification of an animal was positive or negative based on the corpus luteum (CL) blood flow (CLBF) assessed using color Doppler flow imaging at 20 d after timed AI

CLBF classification	Criteria for predictive pregnancy diagnosis based on CLBF[1,2]
Positive	The presence of evident color Doppler signal with colored pixels within the CL that covered most of the edges of the structure and penetrated the luteal tissue toward the center of the gland, demonstrating intense blood supply throughout the entire structure
Negative	No colored pixels on the CL surface or the presence of only a few colored pixels in small parts of the gland primarily on the edges, demonstrating a lack of blood flow to supply the gland's function

[1]The characteristics of the Doppler signal and blood flow observed on the CL structure.

[2]Animals with CL showing intermediate characteristics were classified based in the similarity to 1 of the 2 reference standards.

sign of pregnancy, whereas its absence or small amounts denoted a negative diagnosis. Only colored pixels within or in the immediate vicinity of the CL tissue were considered CLBF. Blood supply to follicles near the CL was not considered CLBF and was carefully avoided.

The ultrasound operator (evaluator no. 1) then recorded the predictive pregnancy diagnosis based only on his subjective, visual evaluation of CLBF (classified as positive or negative). Thirty to 35 d after TAI (d 30–35), blinded to any information on the previous diagnosis (CLBF-d20), evaluator no. 1 performed a final pregnancy diagnosis using conventional B-mode US with a portable scanner (MyLab30Vet Gold) equipped with a 7.5-MHz rectal transducer to visualize the embryonic vesicle and detect the embryo heartbeat, which determined whether each female was classified as pregnant or not pregnant. This final pregnancy diagnosis (referred to as **US-d30**) was considered the diagnostic gold standard because the embryonic heartbeat was visualized.

Within 3 mo of the end of the examinations, evaluator no. 2, blinded to outcomes of evaluator no. 1, watched the cine-loop video clips at the Embrapa Diagnostic Imaging Sector and visually evaluated blood flow on the CL. A predictive pregnancy diagnosis (positive or negative) was also recorded based on his subjective, visual evaluation of CLBF images, which adopted the same criteria used by evaluator no. 1. The final pregnancy outcome by the gold standard (US-d30) was retrospectively compared with the diagnosis based on visual CLBF for both evaluators, and the result from each animal was classified as either correct or incorrect following a criterion previously described by Utt et al. (2009).

Both evaluators had previous experience using a B-mode ultrasound and in-color Doppler flow imaging, were blinded to the animals' reproductive status, and were aware only that those animals were submitted to TAI at 20 d before the ultrasound examination. Each evaluator recorded his diagnoses on independent sheets and had no information on his peer's CLBF classifications.

Blood Sampling and P_4 Analysis

Because determination of plasma P_4 concentrations has also been used as a pregnancy diagnostic test (Thirapatsukun et al., 1978), this pregnancy-related hormone was measured in a randomly assigned subset of 171 females enrolled in this study (71 heifers and 100 cows). The goal was to use plasma P_4 concentration as a reference laboratory diagnostic test for luteal function and to compare the ACC and performance parameters of this test with the CLBF test.

Blood samples were collected by venipuncture of the coccygeal vessels into 4.5-mL tubes containing K3 EDTA (Vacutainer Systems; Becton Dickinson, Sao Paulo, SP, Brazil) just before CLBF-d20 evaluations. Samples were centrifuged at 900 × *g* for 15 min and plasma was harvested, transferred to 1.5-mL tubes, and then stored at −20°C until hormone assays were conducted. Plasma P_4 concentrations were determined using a solid-phase ^{125}I RIA (Coat-Count Progesterone; Siemens Medical Solutions Diagnostic, Los Angeles, CA) at the Sao Paulo State University RIA Laboratory (Sao Paulo, SP, Brazil) following procedures described elsewhere (Viana et al., 2013). The Se was 0.02 ng/mL and the interassay and intraassay coefficients of variation were 2.7 and 9.7%, respectively.

Data Analysis

Retrospective analysis assembled information from both the predictive pregnancy diagnosis (CLBF-d20) and the final pregnancy outcome (US-d30; diagnostic gold standard) for data analyses. Day 20 pregnancy predictions from each of the 2 evaluators were compared with the US-d30 results and categorized as correct or incorrect. Numbers of true positive (**TP**), true negative (**TN**), false positive (**FP**), and false negative (**FN**) results were inserted into a 2 × 2 contingency table (decision matrix) to evaluate the CLBF test performance parameters. Sensitivities and Sp, as defined by Yerushalmy (1947), were calculated to separately evaluate the performance of each evaluator in the CLBF-d20 test. In addition, positive predictive values (**PPV**), negative predictive values (**NPV**), and ACC were also determined for each evaluator. The mean Se, Sp, PPV, NPV, and ACC were calculated using the average TP, TN, FP, and FN between the 2 evaluators. The following equations were used to calculate each parameter: Se = TP/(TP + FN), Sp = TN/(FP + TN), PPV = TP/(TP + FP), NPV = TN/(FN + TN), and ACC = (TP + TN)/n. Prevalence was considered the number of pregnant animals based on the gold standard (US-d30) relative to the number of animals submitted to TAI [i.e., prevalence was equal to pregnancies per AI (**P/AI**)]. Doppler ultrasonographic images representing the 4 CLBF-d20 test categories (TP, FP, FN, and TN) are shown in Figure 1.

Similar retrospective analyses were used for the reference laboratory diagnostic test (plasma P_4 concentration on d 20). Animals that showed plasma P_4 concentration ≥1.0 ng/mL were considered positive and those with P_4 concentration <1.0 ng/mL were considered negative. Day-20 P_4-based results were then compared with the gold standard (US-d30), categorized as correct or incorrect, and the same parameters were

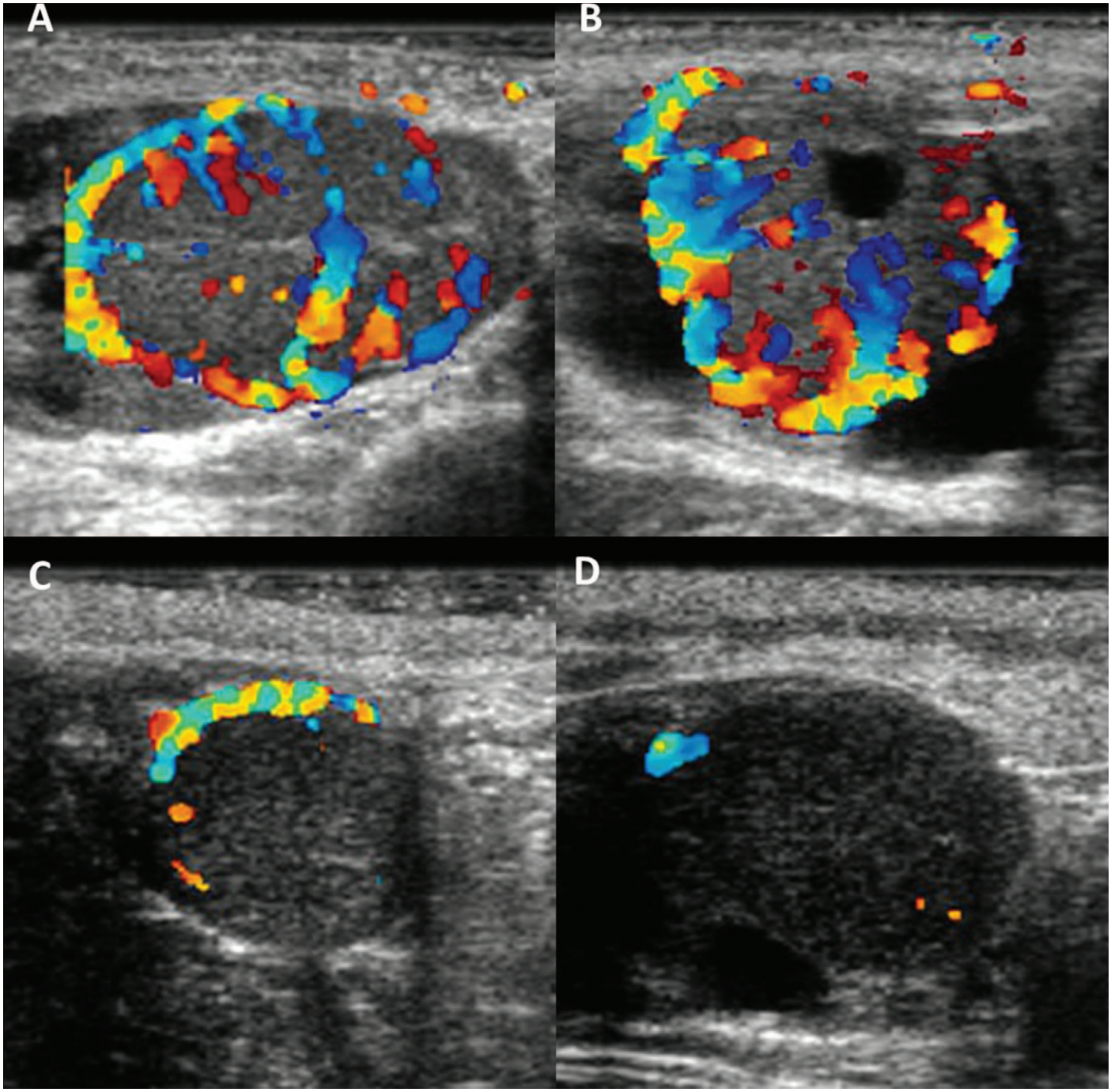

Figure 1. Doppler ultrasonographic images representing the 4 categories observed after pregnancy diagnosis using the corpus luteum blood flow at 20 d after AI (CLBF-d20) test: true positive (A), false positive (B), false negative (C), and true negative (D).

calculated to evaluate the performance of this reference laboratory diagnostic test (Se, Sp, PPV, NPV, and ACC) using the same decision matrix. Proportions of TP, TN, FP, and FN, and Se, Sp, PPV, NPV, and ACC were compared between evaluators (no. 1 vs. no. 2) and between evaluator no. 1 (ultrasound operator) and the reference test (plasma P_4 concentration) using Fisher's exact test. Within each evaluator and plasma P_4 tests, these same endpoints were compared for the effect of parity (heifers vs. cows) using Fisher's exact test.

The CLBF-d20 test was compared with plasma P_4 concentration test results for clinical utility in view of

the Se and Sp observed in each of these tests. For test comparisons, likelihood ratios were calculated to assess probabilities of true results to false results in each test. The likelihood ratio of a positive test (**LR+**) is defined as the probability of a TP (given pregnancy) to a FP (not pregnant). The larger the LR+ the greater the ability of the test to detect pregnancy. The likelihood ratio of a negative test (**LR−**) is the probability of an FN (pregnant) to a TN (not pregnant). The smaller the LR−, the greater the ability to rule pregnancy out. The equations used to calculate these ratios were as follows: LR+ = Se/(100% − Sp) and LR− = (100% − Se)/Sp. The LR+ and LR− were calculated using the Se and Sp from each evaluator in the CLBF test and by using the Se and Sp mean values between evaluators. In addition, LR+ and LR− values were calculated for plasma P_4 concentration tests.

Binomial variables (pregnancy rates and proportions) were analyzed using Fisher's exact test. Pregnancy rates and proportions of TP, TN, FP, FN, Se, Sp, PPV, NPV, and ACC within each evaluator were tested for the effect of parity (heifers vs. cows). The overall pregnancy rate was calculated using the combined data from all of the TAI rounds and both categories (heifers and cows). In addition, TP, TN, FP, FN, Se, Sp, PPV, NPV, and ACC were tested for the effect of evaluator (no. 1 vs. no. 2) and diagnostic test (CLBF-d20 vs. plasma P_4 concentration) using Fisher's exact test. Confidence intervals (95% CI) were calculated for these endpoints using the modified Wald method (Agresti and Coull, 1998).

Kappa values were calculated for each evaluator separately to quantify the degree of agreement between CLBF-d20 and the gold standard (US-d30). The level of agreement between the plasma P_4 concentration test and US-d30 was also quantified. Finally, the inter-rater agreement (between evaluator no. 1 and no. 2) was determined using kappa values. Formulas used to calculate the kappa values are described elsewhere (Noordhuizen et al., 2001) and considered the number of identical results, the proportion of genuine agreement, and corrections for agreement due to chance (expected values for chance alone). If kappa = 0, no agreement exists other than what would be expected by chance. A kappa value of 1 indicates perfect agreement (Martin et al., 1987; Noordhuizen et al., 2001). In the current study, agreements between CLBF-d20 and US-d30 for evaluators no. 1 and no. 2, comparisons between the plasma P_4 test and US-d30, and comparisons between tests (CLBF-d20 vs. plasma P_4) and raters (evaluator no. 1 vs. no. 2) were considered moderate if kappa values were 0.4 to 0.5, good if they were 0.5 to 0.6, and >0.6 was considered to be a high level of agreement, as previously described by Martin et al. (1987). Data were analyzed using the GraphPad InStat software (GraphPad Software Inc., La Jolla, CA), and statistical significance was determined based on a P-value of 0.05.

RESULTS

Synchronization Rate

Of the cows and heifers initially synchronized, 455 of 526 (86.5%) presented with a CL on d 20 after TAI and were used for CLBF evaluations. One heifer developed a luteinized follicular cyst and was excluded from the study. The synchronization efficiency (i.e., the proportion of animals with a CL on d 20) was greater in cows than in heifers (90.9 vs. 80.3%, respectively; $P = 0.0006$). Thus, evaluators examined CL images from 455 females (288 cows and 167 heifers). Each CLBF-d20 examination for prediction of pregnancy using color Doppler imaging was accomplished within <10 s (5 to 7 s) after restraining the animal and detection of the CL using the B-mode US. This short period of time was sufficient for scanning the entire CL structure, visually evaluating the amount of blood flow, and generating a decision on whether the animal was positive or negative based on the Doppler signal.

Pregnancy Rate

The overall rate of pregnancies per AI was 40.3% (212 of 526) and was not affected by parity (42.0 vs. 37.8% for cows and heifers, respectively; $P = 0.36$). If considering only cattle that were successfully synchronized (presenting a CL on d 20), P/AI was 46.6% (212 of 455) when data were combined, 46.2% (133 of 288) in cows, and 47.3% (79 of 167) in heifers. Because parity did not affect P/AI in cattle with CL ($P = 0.84$) or proportions of TP, TN, FP, or FN (Table 2), data from both evaluators and plasma P_4 data were analyzed for efficacy using all of the subjects (455 for the CLBF-d20 test by evaluators no. 1 and no. 2, and 171 for the plasma P_4 concentration test) without distinguishing between parity or category.

Diagnostic Test Performance Parameters

With respect to the performance parameters of the predictive test used in this study (CLBF-d20), the mean values between the 2 evaluators were the following: Se: 99.0%, specificity: 53.7%, PPV: 65.1%, and NPV: 98.5%. The overall accuracy of the test, which is also a measurement of precision, was 74.8%. No differences were observed between evaluators in CLBF-d20 performance endpoints: Se: $P = 0.62$, Sp: $P = 0.58$, PPV: $P = 0.80$, NPV: $P = 0.62$, and ACC: $P = 0.64$. Moreover,

Table 2. Effect of parity (heifers vs. cows) on proportions of true positive (TP), true negative (TN), false positive (FP), false negative (FN), and corpus luteum blood flow at 20 d after AI (CLBF-d20) test performance parameters within each evaluator and within the plasma progesterone (P_4) concentration test results

	CLBF-d20, evaluator no. 1			CLBF-d20, evaluator no. 2			Plasma P_4 concentration test		
Endpoint[1]	Heifers	Cows	*P*-value[2]	Heifers	Cows	*P*-value[2]	Heifers	Cows	*P*-value[2]
TP (%)	46.7	45.5	0.84	47.3	45.8	0.77	45.1	52.0	0.43
TN (%)	29.9	29.2	0.91	29.9	26.7	0.51	25.3	27.0	0.86
FP (%)	22.7	24.6	0.73	22.7	27.0	0.31	29.6	21.0	0.21
FN (%)	0.6	0.7	1.0	0.0	0.3	1.0	0.0	0.0	1.0
ACC (%)	76.6	74.6	0.65	77.2	72.5	0.31	70.4	79.0	0.21
Se (%)	98.7	98.5	1.0	100.0	99.2	1.0	100.0	100.0	1.0
Sp (%)	56.8	54.2	0.78	56.8	49.7	0.29	46.1	56.2	0.39
PPV (%)	67.2	64.8	0.71	67.5	62.8	0.47	60.3	71.2	0.25
NPV (%)	98.0	97.7	1.0	100.0	98.7	1.0	100.0	100.0	1.0
LR+	2.29	2.15	NA[3]	2.32	1.97	NA	1.86	2.29	NA
LR−	0.02	0.03	NA	0.00	0.02	NA	0.00	0.00	NA

[1]Se = sensitivity; Sp = specificity; PPV = positive predictive value; NPV = negative predictive value; LR+ and LR− = likelihood ratios of a positive and of a negative test, respectively.

[2]*P*-values determined using Fisher's exact test.

[3]NA = not applicable.

these parameters did not differ ($P > 0.50$) between the evaluators and the reference laboratory diagnostic test, plasma P_4 concentration. The proportions of TP, TN, FP, and FN were neither different between evaluators ($P > 0.10$) nor between the evaluators and the plasma P_4 concentration test ($P > 0.10$). Lastly, the LR+ and LR− were very similar between the CLBF-d20 and the plasma P_4 concentration test results. All of the performance parameters, proportions, and ratios are shown in Table 3.

The calculated kappa values demonstrated a moderate to good level of agreement between CLBF-d20 and US-d30 for both evaluators and between the plasma P_4 concentration and US-d30 tests. The interrater agreement was high, indicating the reliability of the CLBF-d20 between different investigators (Table 4).

Table 3. Proportions of true positive (TP), true negative (TN), false positive (FP), false negative (FN), and performance parameters for each evaluator (no. 1 and no. 2) of the corpus luteum blood flow at 20 d after AI (CLBF-d20) test, the average of both evaluators (x), and parameters of the plasma progesterone (P_4) concentration test calculated after the final pregnancy diagnosis (gold standard; ultrasonography pregnancy diagnosis d 30 to 35 after AI, US-d30)

	Evaluator[2]					Laboratory diagnostic test, plasma P_4 concentration	
Endpoint[1]	No. 1	95% CI	No. 2	95% CI	x[3]	P_4 test	95% CI
TP [% (no.)]	45.9[a] (209)	41.4–50.5	46.3[a] (211)	41.8–51.0	46.1[a] (210)	49.1[a] (84)	41.7–56.5
TN [% (no.)]	29.4[b] (134)	25.4–33.8	27.9[b] (127)	24.0–32.2	28.7[b] (130.5)	26.3[b] (45)	20.3–33.4
FP [% (no.)]	24.0[c] (109)	20.3–28.0	25.5[c] (116)	21.7–29.7	24.7[c] (112.5)	24.5[c] (42)	18.7–31.5
FN [% (no.)]	0.6[d] (3)	0.13–2.0	0.2[d] (1)	0.01–1.4	0.4[d] (2)	0 (0)[d]	0.0–2.6
Accuracy (%)	75.4[e]	71.2–79.1	74.3[e]	70.1–78.1	74.8[e]	75.4[e]	68.4–81.3
Sensitivity (%)	98.6[f]	95.7–99.7	99.5[f]	97.1–99.9	99.0[f]	100.0[f]	94.8–100.0
Specificity (%)	55.1[g]	48.8–61.3	52.3[g]	46.0–58.4	53.7[g]	51.7[g]	41.4–61.9
PPV (%)	65.7[h]	60.3–70.7	64.5[h]	59.2–69.5	65.1[h]	66.6[h]	58.0–74.3
NPV (%)	97.8[i]	93.5–99.5	99.2[i]	95.3–99.9	98.5[i]	100.0[i]	90.6–100.0
LR+	2.2	NA[4]	2.1	NA	2.1	2.07	NA
LR−	0.03	NA	0.01	NA	0.02	0.00	NA

[a–i]Within a column, proportions with a common superscript did not differ (Fisher's exact test; $P > 0.10$).

[1]PPV = positive predictive value; NPV = negative predictive value; LR+ = likelihood ratio of a positive test; LR− = likelihood ratio of a negative test.

[2]Evaluator no. 1: operator during the ultrasound examinations; performed real-time pregnancy prediction on d 20 and a final pregnancy diagnosis on d 30 to 35. Evaluator no. 2: performed pregnancy prediction by analyzing recorded video clips within 3 mo after the end of the experiment and was blinded to animals' actual pregnancy status and to the results of evaluator no. 1.

[3]Mean of evaluators no. 1 and no. 2.

[4]NA = not applicable.

Table 4. Kappa values calculated to evaluate the relative quality of the 3 diagnostic tests used in this study [corpus luteum blood flow at 20 d after AI (CLBF-d20), ultrasonography pregnancy diagnosis d 30 to 35 after AI (US-d30), and plasma P_4 concentration]

	Agreement between tests			
Comparison	Kappa[1]	SE[2]	95% CI	Level of agreement[3]
CLBF-d20 test vs. US-d30, evaluator no. 1	0.52	0.03	0.45–0.59	Good
CLBF-d20 test vs. US-d30, evaluator no. 2	0.50	0.03	0.43–0.57	Moderate to good
d-20 plasma P_4 concentration vs. US-d30, laboratory test reliability	0.51	0.06	0.40–0.62	Good
Evaluator no. 1 vs. evaluator no. 2, interrater reliability on CLBF-d20	0.91	0.02	0.87–0.95	High

[1]Quantified agreement between results (pregnancy outcomes) of different tests (CLBF-d20, plasma P_4 concentration, and gold standard US-d30) and with different testers (evaluators no. 1 and no. 2).

[2]Standard error of the kappa value.

[3]A kappa value of 1 indicates a perfect agreement, whereas a value of 0 denotes no agreement at all, beyond chance (Noordhuizen et al., 2001). Kappa values of between 0.4 and 0.5 indicate moderate agreement, 0.5 and 0.6 good agreement, and >0.6 a high level of agreement (Martin et al., 1987).

Seventeen observed disagreements existed between evaluators no. 1 and no. 2, which represented 3.7% of the 455 examinations.

DISCUSSION

Early detection of nonpregnant females is mandatory for optimal reproductive management on dairy farms and for reducing the number of days open and calving intervals. In the current study, as suggested by other authors (Utt et al., 2009; Herzog et al., 2010; Quintela et al., 2012), we hypothesized that luteal blood flow, assessed using color Doppler ultrasound, would be a reliable diagnostic test of pregnancy when performed at 20 d after TAI. The novelty in this study was that the diagnoses were based only on visual evaluations of CLBF, a subjective yet practical approach to determine whether the animal was pregnant or not. Indeed, we have demonstrated that the CLBF-d20 test was characterized by high Se (99%), medium Sp (53.7%) and, most importantly, high NPV (98.5%). Examinations were performed within a <10-s interval (5 to 7 s) after detecting the CL, the diagnostic criteria were clear and straightforward, results were consistent between rounds of TAI and between evaluators, and performance parameters were similar to the laboratory diagnostic test (plasma P_4 concentration), an indicator of CL function. With respect to accuracy, CLBF-d20 was as accurate as plasma P_4 measurements for early diagnosis of pregnancy on d 20 (74.8 vs. 75.4%, respectively; $P > 0.10$), which were later determined using the gold standard (US-d30).

Our findings are apparently in contrast to conclusions drawn by a previous study that also analyzed luteal blood flow in cattle and reported that this endpoint was not an appropriate diagnostic tool for the early detection of pregnancy (Herzog et al., 2011). This same study, however, observed significant differences in luteal blood flow between cows later diagnosed as pregnant versus nonpregnant cows, which is similar to the results of our study. The apparent divergent conclusions of the present study and that previous study were most likely related to the period within which pregnancy prediction was performed. Herzog et al. (2011) analyzed luteal blood flow on d 15 and 18, whereas the current study analyzed CLBF a few days later (d 20) when the differences between pregnant and nonpregnant animals are expected to be greater. The previously reported major concern on the feasibility of an early pregnancy test was the high variation between animals (Herzog et al., 2011). Conversely, the impact of this variation is expected to decrease as differences in CLBF between pregnant and nonpregnant animals increase. Therefore, both studies agree that CLBF is reduced in nonpregnant cows but we also demonstrated that the CLBF-d20 test is feasible for the early detection of nonpregnant cattle when performed at a specific time point after AI (d 20).

In this study, we could predict pregnancy based on the CLBF at 20 d after AI with high Se and a medium Sp. Consistent with previous studies, the accuracy of the CLBF-d20 diagnoses was higher for negative (NPV) than positive (PPV) cases (98.5 vs. 64.8%, respectively; $P < 0.001$). A greater concern was the likelihood of correctly identifying cattle as truly not pregnant to ensure that this test would be actually useful in an intensive reproductive management program. In view of the inconsistencies of measuring only quantities of FP and FN, the NPV was considered the most important performance parameter for the CLBF-d20 test because it represented the proportion of animals testing negative (by CLBF-d20) that were truly not pregnant (by gold standard; US-d30). Our results verified that the CLBF-d20 correctly identified a negative result approximately 98.5% of the time. Because the major reason for diagnosing pregnancies early is to correctly

identify nonpregnant animals (Fricke and Lamb, 2005; Romano et al., 2006), evaluations of CLBF 20 d after AI can, therefore, be considered to be a reliable tool for that purpose.

It is noteworthy that the FN diagnoses, although very low (0.6 vs. 0.2%, for evaluator no. 1 and no. 2, respectively), occurred during the first round of TAI, whereas no FN were detected in the other 4 rounds. Moreover, all of the observed disagreements between evaluators (3.7%; 17 of 455) occurred when CL showed transitional blood flow patterns (moderate to low CLBF). This raises the question of the role of the operator's experience of diagnosing with CLBF-d20. We observed that as the operators familiarized themselves with the process and consolidated mental standards and thresholds for the subjective evaluations of blood flow in the CL, negative diagnoses become very reliable (i.e., FN results were rare or even absent). Operator proficiency is required for high ACC and efficiency in pregnancy diagnosis by transrectal US (Fricke, 2002). Indeed, as we observed in the current study, this proficiency was also necessary for Doppler ultrasound to enable the evaluation of CLBF at d 20 after AI.

The higher accuracy of CLBF-d20 for detecting nonpregnant than for detecting pregnant animals was expected and is consistent with previously described findings (Quintela et al., 2012). The amount of CLBF on d 20 is an indirect indication of the success or failure of the establishment of pregnancy, which may not be confirmed at a later stage due to embryonic losses occurring early after maternal recognition of pregnancy (d 21 to 28) but before d 30 to 35. Incorrect CLBF-d20 diagnoses may also occur in estrous cycles with extended luteal phases (i.e., delayed CL regression), the occurrence of which has previously been described in dairy cows (Giordano et al., 2012). That study also observed that P_4 concentrations before d 22 after TAI were not different between pregnant cows and those with an extended luteal phase most likely because the latter experienced embryonic loss after the period of maternal recognition of pregnancy. Although the experiment reported here was not designed to identify early pregnancy losses, it is reasonable that embryo mortality rather than misdiagnosis may have accounted for some of the observed FP. This result was similar to that reported for early pregnancy diagnosis using grayscale US on d 24 to 26 of gestation (Quintela et al., 2012). Embryo mortality during early stages of pregnancy is particularly common in dairy cattle (Smith and Stevenson, 1995; Chebel et al., 2004), and its incidence was estimated to be 20% at approximately d 28 after AI (Moore and Thatcher, 2006), whereas the rate of late embryonic losses was 12.8% from approximately 28 to approximately 45 d of gestation (reviewed by Santos et al., 2004). The theory that a significant number of the CLBF-d20 FP observed in the present study were due to embryonic mortality or delayed luteolysis, or both, is supported by the consistency with the PPV of the plasma P_4 concentration test, indicating that those CL remained functional regardless of confirmation of pregnancy using US-d30. Moreover, the 10-d interval between d-20 evaluations and US-d30 may have factored in the observed medium specificity of the CLBF-d20 test. In summary, any of the currently available early pregnancy diagnostic tests may have shown some FP results either due to embryo mortality or late luteolysis rather than misdiagnosis. The proportion of FP (24.7%) using the CLBF-d20 test was, therefore, expected and was considered acceptable.

A pregnancy diagnostic test must be feasible in a practical routine in addition to being reliable and accurate. Blood collection and laboratory assays to determine plasma (Thirapatsukun et al., 1978) or milk (Pennington et al., 1976; Gowan and Etches, 1979) P_4 concentrations or to detect specific pregnancy-associated plasma proteins (Humblot et al., 1988; Silva et al., 2007a) have been used previously for pregnancy diagnosis in dairy cattle. Results, however, were contradictory and, therefore, of limited potential use in large herds. Moreover, the need for blood sampling, centrifugation, plasma separation, and lastly the laboratory assay itself made these tests complex, time consuming, and expensive. In the present study, the accuracy of the plasma P_4 assay for pregnancy diagnosis was similar to the CLBF-d20 evaluation. In addition, grayscale B-mode US examinations for pregnancy before d 26 to 28 have also been described as time consuming, of only fair reliability, and of limited accuracy (Pieterse et al., 1990; Romano et al., 2006; Quintela et al., 2012). In addition to the requirement for sketching the ovaries to detect the CL and the uterine horns to detect the embryonic vesicle, the presence of uterine mucus may hamper the operator in cows that are in proestrus or estrus phases (Pieterse et al., 1990; Kastelic et al., 1991; Quintela et al., 2012). Thus, Romano et al. (2006) observed that maximum Se and NPV of B-mode US are achieved only at d 26 after AI in heifers and d 29 in cows. When compared with the previous alternatives (plasma P_4 and B-mode US), color Doppler flow imaging was at least as accurate in addition to being less complex and much more rapid (<10 s for each CLBF evaluation) for early diagnosis of pregnancy in cattle, which are desirable attributes for an on-farm management routine. We observed that color Doppler to assess CLBF on d 20 is a quick, straightforward, reliable approach that enables the detection of a high percentage of nonpregnant animals, thus enabling early and real-time decisions on whether resynchronization,

treatment, or any other adjustment is required for the reproductive management of those animals. Fricke and Lamb (2005) have suggested that strategies by which to integrate US and ovulation synchronization protocols should be developed and optimized. The results of the current study demonstrate that in the near future, not only grayscale US but also color Doppler flow imaging should be incorporated into a proactive reproductive management protocol because of its ability to diagnose very early pregnancies and to enable early resynchronization.

Different groups have evaluated CLBF in cattle by measuring the Doppler signal area, the ratio Doppler signal area:CL area, or colored pixel intensity in the Doppler area in previously selected and recorded images (Acosta et al., 2002; Herzog et al., 2010; Shrestha et al., 2010). Although these approaches resulted in objective measures of CLBF, the necessity of postacquisition image processing limits real-time decisions. The approach used in the present study, although subjective, is much less time consuming and easier to incorporate into the reproductive management routine. Furthermore, a strong agreement between objective and subjective evaluations of CLBF has previously been reported (Ginther et al., 2007). We also verified that results were similar when CLBF-d20 was evaluated together with the examination itself (evaluator no. 1) or when it is evaluated at a later time point, by watching recorded videos (evaluator no. 2). This feature may be advantageous for future research into ovarian physiology because a large number of subjects may be scanned at a time and the images can be analyzed later by several other investigators.

Together, the results in this study of NPV, PPV, ACC, and time required to perform the CLBF-d20 test strongly suggest that color Doppler imaging has great potential as a pregnancy diagnostic tool in the reproductive management of cattle. Taking into account the Sp and NPV observed in the current study, a CLBF-d20 test may be considered promising for detecting more than half (Sp = 53.7%) of the nonpregnant females a few days earlier than grayscale US (i.e., 20 d after AI). In addition, in view of the number of animals diagnosed as without a CL on d 20 and those with any pathologic condition (e.g., follicular luteinized cyst), the value of the CLBF-d20 evaluations appear to be even greater as a diagnostic test to early detect nonpregnancy. Those females could be either treated or resynchronized (i.e., an earlier decision could be made for those animals that presented synchronization failures). One must also consider that some of the cattle may have had short cycles (<20 d in length). These animals most likely did not present an evident CL with adequate blood flow on d 20 or presented with a corpus hemorrhagicum. In either situation, they were correctly classified as nonpregnant due to the lack of CLBF or lack of a clearly defined CL and were resynchronized.

In the particular case of dairy farms, color Doppler flow imaging for very early (d 20) detection of nonpregnant cows may represent a substantial improvement in the reproductive management, because the efficiency of estrus detection is a major issue and P/AI (prevalence of pregnancy) is approximately 35 to 45% (reviewed by Lucy, 2001). The integration of ultrasound into dairy management strategies has been suggested by Fricke (2002). This author recommended the identification of nonpregnant cows using B-mode US on d 26 after AI and GnRH treatment for resynchronization using the Ovsynch protocol (Pursley et al., 1995). A more aggressive approach would be a GnRH injection at 19 d after AI, pregnancy diagnosis by US on d 26, and TAI on d 28. The former strategy would result in an average interval between AI of 35 to 36 d and of 28 d in the latter (Fricke, 2002). If the CLBF-d20 test was used as described in the current study, the interval between services could be reduced to 29 to 30 d (depending upon the TAI protocol) or, if using the more aggressive approach, to 22 to 23 d in more than half (Sp = 53.7%) of the females that failed to conceive after the initial TAI. Furthermore, in farms with a low prevalence of pregnancy (low P/AI), NPV tends to increase and is even higher, thereby increasing the impact of early pregnancy diagnostic tools. These estimated 5-d reduction in the interinsemination interval may or may not be adopted depending on the management of each particular farm. Nonetheless, we believe that the use of a CLBF-d20 test as described in this study could be a very useful strategy by which to reduce days open and to increase reproductive efficiency in high-producing dairy herds.

Further research should provide additional options for incorporating CLBF-d20 evaluations into the reproductive routine. For example, CLBF-d20 can be used to select nonpregnant animals for early resynchronization with a consequent reduction in the interinsemination intervals. Alternatively, new resynchronization protocols may be developed to initiate even earlier where the second TAI occurs or not based on CLBF-20d results. In this case, AI would occur at the same time as the expected natural return to estrus, with the advantage that no estrus detection would be required. A potential limitation of this approach would be that fertility appears to be reduced when the TAI protocol is initiated in the absence of a CL (i.e., in a low-P_4 environment; Fricke et al., 2003; Silva et al., 2007b). Thus, strategies by which to rapidly resynchronize cattle after a diagnosis of nonpregnancy using the CLBF-d20 test must be developed and tested. Certainly, this type of approach

must be investigated for feasibility but evokes a great deal of enthusiasm.

CONCLUSIONS

A single visual evaluation of CLBF using color Doppler flow imaging offered high ACC in predicting nonpregnant females at 20 d after TAI. Corpus luteum blood flow at 20 d after AI evaluations can be reliably used as a pregnancy diagnostic test and show high Se and NPV but the operator's experience and definition of clear criteria for positive and negative diagnoses are of great importance for successful outcomes. Further studies on strategies by which to incorporate Doppler CLBF-d20 evaluations into aggressive TAI reproductive management may contribute substantially to reducing days open and calving intervals, particularly in dairy cattle. We conclude that as the primary objective of early pregnancy diagnosis is to identify nonpregnant animals for resynchronization, the visual evaluation of CLBF at 20 d after TAI provides a quick, reliable, and consistent pregnancy diagnostic test.

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