



Early pregnancy diagnosis at 21 days post artificial insemination using corpus luteum vascular perfusion compared to corpus luteum diameter and/or echogenicity in Nelore heifers



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ABSTRACT

The objective was to compare the use of corpus luteum (CL) vascular perfusion to CL diameter and/or echogenicity to diagnose pregnancy at 21 d after timed-AI. Ovaries of Nelore heifers were assessed using ultrasonography in B-mode and color Doppler simultaneously 21 d after timed-AI ($n = 113$). Objective evaluations were performed using an image processing software to extract the number of colored pixels (ColorPix), diameter (mm) and echogenicity/mm² (EchoPix) of the CL. Subjective evaluations of the CL were performed by five evaluators using scores of estimated vascular perfusion area of color Doppler scan videos and estimated CL size and qualitative echogenicity of B-mode scan videos. The reference pregnancy diagnosis was performed 33 d after timed-AI using an ultrasonic device. Corpus luteum ColorPix, diameter and EchoPix were highly correlated ($P < 0.001$) with pregnancy. Pregnancy diagnosis accuracy, sensitivity, and negative predictive value were not different for CL ColorPix and diameter and was less with use of EchoPix compared to the other parameters. Size and vascular perfusion scores were correlated to the greatest extent (0.88–0.94) with the respective objective values within evaluator. The results from the ROC curve analysis indicated a smaller area under the curve for qualitative echogenicity compared to CL size and vascular perfusion. Corpus luteum vascular perfusion was the only subjective evaluation that when assessed there were no false negative pregnancy diagnoses. In conclusion, the use of the objective CL diameter resulted in the same efficacy as CL vascular perfusion evaluations for early pregnancy diagnosis in Nelore heifers.

1. Introduction

The early identification of non-pregnant cows and heifers allows for earlier re-insemination, increases the service rate and reduces the calving interval in commercial tropical cow-calf operations (Sá Filho et al., 2014; Crepaldi et al., 2017). Methods of early

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pregnancy diagnosis should be simple to perform, provide an immediate result, and be economical while having a precise accuracy if there is going to be commercial use of the technique (Fricke et al., 2016). The absence of false negative is a key factor when choosing an early pregnancy diagnosis method due to the abortion risk when there is use of hormonal treatments in cows that are pregnant but that are misdiagnosed as being non-pregnant (Pugliese et al., 2017). Indirect methods for early pregnancy diagnosis such as interferon-stimulated genes [ISGs] (Green et al., 2010), pregnancy associated-glycoproteins [PAGs] (Ricci et al., 2015; Martins et al., 2018), and plasma concentrations of progesterone (Chebel et al., 2003) have an acceptable accuracy (> 80%). Because the indirect methods do not provide an instantaneous result (cow-side test), the use of these techniques in beef cattle reproductive management programs is limited.

The corpus luteum (CL) vascular perfusion score as assessed using color Doppler ultrasonography was correlated to CL functionality, circulating concentrations of progesterone, and has been used as an early pregnancy diagnosis in cattle between 20 and 22 d post-AI (Matsui and Miyamoto, 2009; Pugliesi et al., 2014). This test allows an immediate early pregnancy diagnosis, with an acceptable occurrence of false negative diagnoses with similar accuracy (80%–95%) when compared to ISGs (Matsui and Miyamoto, 2009; Pugliesi et al., 2014). The cost of a device equipped with Doppler is about three to four times greater when compared to a machine equipped only with the brightness mode [B-mode] (Pugliesi et al., 2017). In addition, the varying machine settings used and lack of calibration makes the standardization of this technique complicated and may impair the replication of this evaluation in the field (Thijssen et al., 2011; Scully et al., 2015). The use of subjective scores, however, may facilitate the interpretation of results (Ginther, 2007).

Results of previous research indicated that the use of CL and uterine morphological characteristics using B-mode ultrasonography resulted in an acceptable accuracy ($\geq 85\%$) to determine pregnancy on d 18, 20 and 22 after ovulation in Holstein heifers (Kastelic et al., 1989). The use of this approach could result in a similar pregnancy diagnosis accuracy (80%–95% compared with using the CL vascular perfusion approach with color Doppler ultrasonography (Pugliesi et al., 2017). There, however, is a lack of research directly comparing the efficiency of early pregnancy diagnosis using B-mode ultrasonography for assessing CL morphological characteristics with the technique of CL vascular perfusion using color Doppler.

The present study was conducted to compare the efficacy when there was use of the CL vascular perfusion technique from determining CL diameter and/or echogenicity to determine pregnancy at 21 d post-TAI in Nelore heifers. The hypothesis was that for early pregnancy diagnosis at 21 d after TAI the assessment of CL vascularity using color Doppler ultrasonography would have a similar accuracy and sensitivity compared with CL diameter and/or echogenicity evaluation using B-mode ultrasonography. The use of B-mode ultrasonography for early pregnancy diagnosis would provide for an immediate and cost-effective response that could be routinely used in beef cattle reproductive programs.

2. Materials and methods

2.1. Animals, location, and reproductive management

Data collection was conducted from December 2017 to February 2018 on a commercial cow-calf operation in Brazil. Nulliparous Nelore heifers ($n = 76$) with a body weight average of 333.4 ± 3.3 kg and that were considered eligible for insemination were used in this study. Heifers were maintained in a *Brachiaria brizantha* pasture with *ad libitum* access to water and mineral salts. All animal handling and experimental procedures were approved by the Animal Use and Ethics Committee of the Instituto de Veterinária of the Universidade Federal Rural of Rio de Janeiro (Protocol #: 1730040918).

Heifers were estrous synchronized to receive first timed artificial insemination (TAI; $n = 76$) using a similar protocol as that of Sá Filho et al. (2008). The protocol was initiated on d 0 with the insertion of a third use intravaginal progesterone (P4) device (CIDR, Zoetis, São Paulo, SP, Brazil) and i.m. administration of 2 mg of estradiol benzoate (Sincrodiol, Ourofino, Cravinhos, SP, Brazil). On d 8, the P4 device was removed, and 150 μ g of d-cloprostenol (PGF_{2 α} ; Veteglan, Hertape Calier, Juatuba, MG, Brazil), 1 mg of estradiol cypionate (ECP, Zoetis, São Paulo, SP, Brazil) and 300 IU of equine chorionic gonadotropin (eCG; Folligon, MSD, São Paulo, SP, Brazil) were administered i.m. On d 10, there was a TAI using semen from the same sire and the same processing batch. For all heifers, there was initiations of an estrous resynchronization treatment 12 d after the first TAI with the insertion of a third-use intravaginal P4 device. The P4 device was removed 7 d later, and 300 IU of eCG was administered i.m. All heifers had ovarian evaluations 21 d after first TAI by an experienced technician using a color Doppler ultrasonic equipment (Z5 Vet, Mindray, Shenzhen, China) with a 5 to 7.5 MHz frequency linear array transducer. Heifers that had a CL with $\leq 25\%$ vascular perfusion were considered to not have a functional CL and to be non-pregnant (Pugliesi et al., 2014). These heifers ($n = 37$) received 100 μ g of GnRH (Fertagyl, MSD, São Paulo, SP, Brazil) and there was a second TAI immediately after the time of the ultrasonography examination.

Pregnancy diagnosis was conducted by one technician 33 d after first and second TAI in all heifers using the same ultrasonic device on B-mode. Pregnancy was confirmed by the presence of an embryo with a heartbeat. This pregnancy diagnosis was considered the reference (“gold standard”) test.

2.2. Experimental design

Ultrasonography examination of the ovaries was performed on all heifers 21 d after the first and second TAI ($n = 113$) by one experienced veterinarian using the previously described machine. The ultrasonic equipment was operated on Dual-Synch mode displaying two simultaneous images of the same anatomical area, one on color Doppler mode and another on B-mode. Settings for color Doppler examination were as follow: 5.0 MHz frequency, 54 color gain, 222 color wall filter, and 1.0 kHz pulse repetition

frequency. Ultrasonic B-Mode settings used were: 7.5 MHz, 7.4 cm depth, 69 gain, 15 frame rate, and 145 dynamic range. Videos of ovarian assessments were recorded and used for later evaluation of objective and subjective CL characteristics.

2.2.1. Objective evaluation of CL vascular perfusion, diameter, and echogenicity

For each color Doppler ultrasonographic video recorded, a single screenshot of the CL with the apparent maximum number of color pixels was selected and transferred using a picture processing software (Adobe Photoshop - CS4 version 11.0, Adobe Systems, San Jose, CA). This software was used to obtain an image of the CL area with only red and blue pixels similar to the color scale of the ultrasonic console display. Red and blue pixel numbers of the luteal area (ColorPix: luteal vascular perfusion) were counted on each image using the ImageJ software version 1.45 s (National Institutes of Health, Bethesda, MD). For calculation of the largest CL diameter, an image with the apparent greatest CL size was selected from the B-mode ovarian assessment videos of each examination. The ImageJ software was used to measure the largest CL diameter (d) based on the mean of two perpendicular measurements. A similar method to obtain ColorPix was used to calculate echogenicity using B-mode scan videos. An image of the CL with the apparent greatest echogenicity was selected and saved. The image was transferred using the picture processing software to obtain an image of the CL area with only gray pixels similar to the grayscale displayed on the ultrasonic console display. ImageJ was used to calculate the number of pixels within each shade of gray [white = 0 to black = 255; (Scully et al., 2015)]. Total CL echogenicity was calculated using the sum of shades of gray for each pixel in the total luteal area (mm^2 ; EchoPix). Total luteal area for each CL was calculated using the equation: $\pi(d/2)^2$ (Martins et al., 2011). The minimum values obtained for each objective measurement of CL vascular perfusion (ColorPix), diameter (mm) and echogenicity/ mm^2 (EchoPix) in pregnant heifers at d 33 after-TAI were used as a cut-off value to determine pregnancy at d 21 post-TAI for these parameters.

2.2.2. Subjective evaluation of CL vascular perfusion, size, and echogenicity

Corpus luteum vascular perfusion, size, and echogenicity were evaluated subjectively by five experienced veterinarians blinded to any additional information for each ovarian ultrasonography video. Color Doppler ultrasonographic examination videos were used to assess CL vascular perfusion. Subjective scores were used to categorize CL vascular perfusion (1 = 0%–25 %; 2 = 26%–50 %; 3 = 51%–75 %, and 4 = 76%–100 % of color pixels inside the luteal area; Fig. 1.). Heifers with vascular perfusion scores ≥ 2 were considered pregnant at 21 d post-TAI. This criterion was previously reported for assessing CL vascularity at d 20 post-AI using color Doppler ultrasonography examinations in Nelore cows, which resulted in a 100% sensitivity and negative predictive value (Pugliesi et al., 2014). To determine if the evaluators were influenced by CL size during categorization of CL vascular perfusion, there was also calculation of the average of ColorPix determinations per total luteal area (ColorPix/ mm^2) for each score.

Videos of B-mode ultrasonography assessments were used to evaluate CL size and echogenicity. Corpus luteum size was classified as small (< 13 mm), medium (13–19 mm), and large (> 19 mm). Heifers that had a small CL were considered to be non-pregnant. Corpus luteum echogenicity was classified into two scores (1 = few nonechogenic points; and 2 = maximum nonechogenic points; Fig. 1.). Only heifers with CL echogenicity classified as having the maximum number of nonechogenic points (2) were considered pregnant. Pregnancy status of heifers was also determined by use of a combination of the subjective scores of CL size and echogenicity. In this case, only heifers that had medium or large CL size combined with maximum nonechogenic points (2) were considered pregnant.

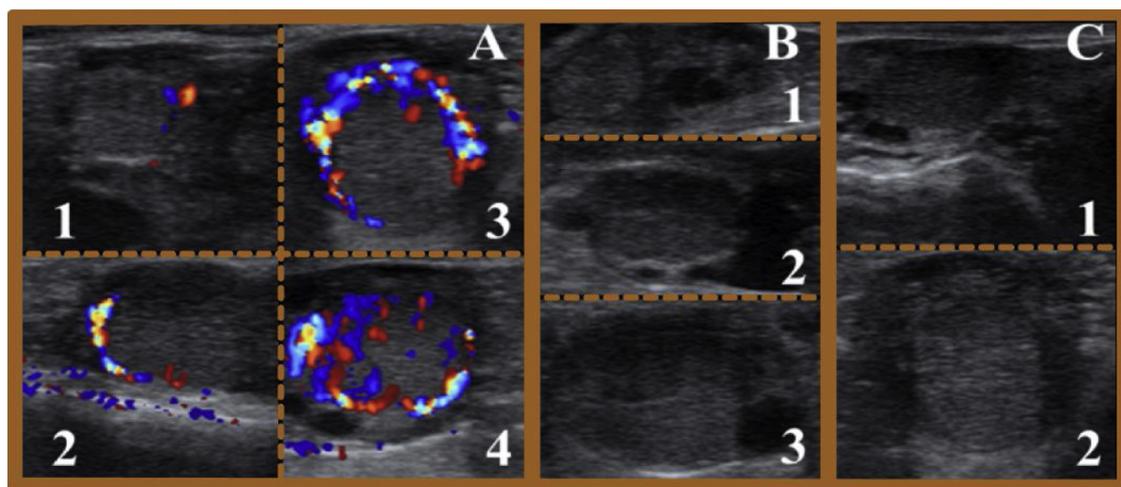


Fig. 1. Illustration of the subjective evaluation of CL morphology in Nelore heifers using ultrasonography with color Doppler and B-mode; (A) estimated CL vascular perfusion scores (1 = 0%–25%; 2 = 26%–50%; 3 = 51%–75%, and 4 = 76%–100% of color pixels inside luteal area); (B) estimated CL size scores: 1 = small (≤ 13 mm), 2 = medium (13–19 mm), and 3 = large (> 19 mm); (C) estimated luteal echogenicity scores using the estimated echogenicity of CL: 1 (few nonechogenic points) and 2 (maximum nonechogenic points).

Table 1

Pregnant and non-pregnant heifers based on predictive (early pregnancy diagnosis) and definitive (reference pregnancy diagnosis) diagnostics; Formulae used to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of early pregnancy diagnosis compared to the reference pregnancy diagnosis were: sensitivity = $[a/(a + c)] \times 100$; specificity = $[d/(b + d)] \times 100$; PPV = $[a/(a + b)] \times 100$; NPV = $[d/(c + d)] \times 100$; Accuracy = $[(a + d)/(a + b + c + d)] \times 100$.

		Reference Pregnancy Diagnosis		Total
		Pregnant	Not Pregnant	
Early Pregnancy Diagnosis	Pregnant	a	b	(a + b)
	Not Pregnant	c	d	(c + d)
	Total	(a + c)	(b + d)	(a + b + c + d)

a = heifers pregnant at the time of early pregnancy diagnosis and pregnant at the time of the reference pregnancy diagnosis.

b = heifers pregnant at the time of early pregnancy diagnosis and non-pregnant at the time of the reference pregnancy diagnosis.

c = heifers non-pregnant at the time of early pregnancy diagnosis and pregnant at the time of the reference pregnancy diagnosis.

d = heifers non-pregnant at the time of early pregnancy diagnosis and non-pregnant at the time of the reference pregnancy diagnosis.

2.3. Statistical analysis

The statistical analyses were performed using SAS (Statistical Analysis System, version 9.4). Predicted probability of pregnancy at d 33 post-TAI based on each objective CL measurement at d 21 post-AI (ColorPix, diameter, or EchoPix) was calculated using logistic regression utilizing the LOGISTIC procedure. This same analysis was also performed using objective measurements of CL diameter and EchoPix in the same model. The CORR procedure was used for determinations of Pearson correlation coefficients for CL subjective scores of vascular perfusion, size, and echogenicity performed by the five evaluators and the objective values of ColorPix, diameter, and EchoPix, respectively. The differences in the proportion of false pregnancy diagnosis (positive and negative) at d 21 post-AI using subjective CL vascular perfusion, size, and echogenicity scores were analyzed by chi-squared using the PROC FREQ. The effect of evaluator on false pregnancy diagnosis was analyzed with PROC GLIMMIX. Because there was no evaluator effect on the proportion of false diagnoses for the different subjective scores evaluated, the pregnancy diagnosis of each method of each evaluator was considered one study observation (113 exams \times 5 evaluators; $n = 565$) for sensitivity, specificity, positive (PPV) and negative predictive values (NPV), and accuracy analyses (Table 4). Sensitivity, specificity, PPV, NPV, and accuracy comparing the early pregnancy diagnosis using CL objective and subjective parameters at 21 d post-TAI to the reference pregnancy diagnosis at d 33 post-TAI were calculated using the formulae in Table 1. Differences in sensitivity, specificity, PPV, NPV, and accuracy of the different early pregnancy diagnosis methods were analyzed using a chi-squared test utilizing the PROC FREQ. Receiver operating characteristic (ROC) curve analysis was performed using PROC LOGISTIC, and area under the curve (0–1) was generated based on the sensitivity and specificity of each method of early pregnancy diagnosis. To test if evaluator was influenced by CL size during categorization of CL blood vascularization (scores 1–4), there was assessment of the effect of score, evaluator and the interaction on average ColorPix/mm² using PROC MIXED of SAS. There was log transformation of ColorPix/mm² to normalize the residuals. Probability values were considered significant when the *P* value was ≤ 0.05 .

3. Results

Corpus luteum objective evaluation of ColorPix, diameter and EchoPix at d 21 post-TAI was positively correlated ($P < 0.001$) with the reference pregnancy diagnosis on d 33 post-TAI ($r = 0.65, 0.70, \text{ and } 0.41$, respectively). When CL diameter and EchoPix were used in the same model, the positive correlation was less ($r = 0.53$) compared to only CL diameter in the model. The ColorPix and CL diameter method of early pregnancy diagnosis had a 100% NPV and sensitivity because of the absence of false negative diagnoses, whereas the EchoPix had a lesser NPV and sensitivity (Table 2). For the ColorPix and CL diameter method of early pregnancy diagnosis, there was a similar accuracy and greater accuracy, respectively, compared with only using the EchoPix

Table 2

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for corpus luteum (CL) objective values as a method of early pregnancy diagnosis at 21 d post-TAI in Nelore heifers; Cut-off point used for pregnancy diagnosis was the greatest CL objective value for each variable in heifers that were non-pregnant heifers at d 33 post-TAI.

	ColorPix ¹	CL diameter	EchoPix ²
Sensitivity	100% (47/47) ^a	100% (47/47) ^a	74.5% (34/47) ^b
Specificity	81.8% (54/66)	77.3% (51/66)	81.8% (54/66)
PPV	79.7% (47/59)	75.8% (47/62)	74.5% (35/47)
NPV	100% (54/54) ^a	100% (51/51) ^a	81.8% (54/66) ^b
Accuracy	89.4% (101/113) ^a	86.7% (98/113) ^a	78.8% (89/113) ^b

^{a,b} Percentages within a row with different letters differ ($P < 0.05$).

¹ ColorPix: total number of colored pixels in the CL area.

² EchoPix: pixel intensity in grayscale/CL area (mm²).

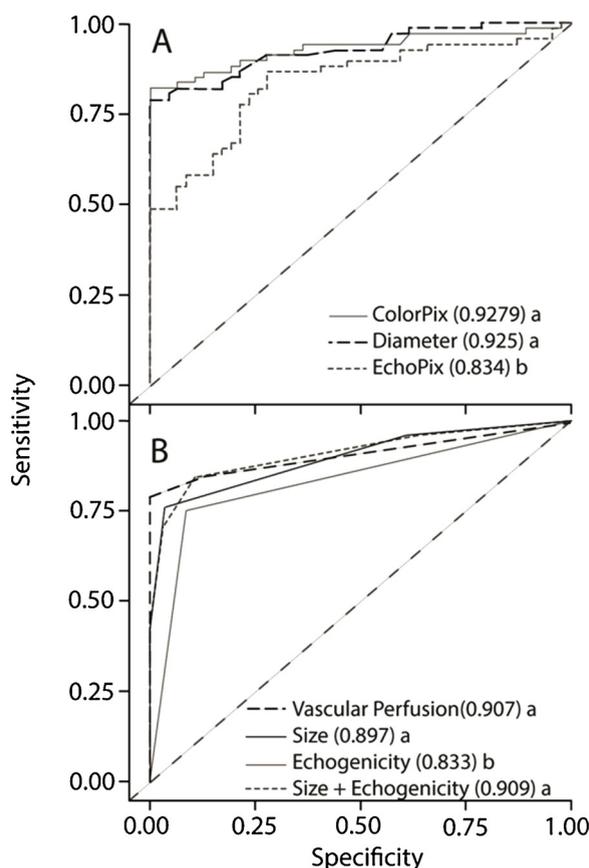


Fig. 2. ROC curve analyses comparing the efficiency of using (A) objective evaluations of ColorPix (total number of colored pixels in the CL area), EchoPix (pixel intensity in grayscale/luteal area mm^2), and CL diameter (mm) or (B) subjective score evaluations of the luteal vascular perfusion, echogenicity, CL size, and CL size + luteal echogenicity for determining pregnancy at 21 d post-TAI based on the reference pregnancy diagnosis at 33 d post-AI; Legend names with different letters indicate differences ($P < 0.05$).

approach for early pregnancy diagnosis. With use of the early pregnancy diagnosis methods for the objective assessment of the CL (ColorPix, CL diameter, and EchoPix), there was a similar specificity and PPV (Table 2). The area under the curve was not different with use of the ColorPix and CL diameter methods for pregnancy diagnosis (Fig. 2).

The CL subjective scores of vascular perfusion, size, and echogenicity estimated by five evaluators were positively correlated with values for ColorPix, CL diameter, and EchoPix, respectively (Table 3). The correlation between vascular perfusion score and ColorPix or between estimated CL size score and CL diameter was large when data from all evaluators were assessed ($r \geq 0.88$; Table 3). Average mean ColorPix/ mm^2 was different ($P < 0.001$) for different scores (Score 1 = 1.1 ± 0.1 ; Score 2 = 8.2 ± 0.4 ; Score 3 = 10.8 ± 0.2 ; and Score 4 = 13.4 ± 0.4 ColorPix/ mm^2). There, however, was no effect of evaluator ($P = 0.18$) and no interaction effect between scores and evaluator ($P = 0.42$) on ColorPix/ mm^2 , indicating that all evaluators accurately assessed the percentage of blood perfusion of the CL and were not influenced by CL size during blood perfusion categorization.

In addition, there was no effect of evaluator on the proportion of false diagnoses using early pregnancy diagnosis for the different

Table 3

Pearson correlation coefficient (r) between objective values ColorPix, EchoPix and CL diameter with subjective scores assessed by five different evaluators.

	Evaluators				
	1	2	3	4	5
ColorPix ¹	0.90	0.88	0.90	0.90	0.91
CL diameter	0.94	0.92	0.92	0.92	0.91
EchoPix ²	0.70	0.68	0.70	0.69	0.64

All values were significant $P < 0.0001$.

¹ ColorPix: total number of colored pixels in the CL area.

² EchoPix: pixel intensity in grayscale/CL area (mm^2).

Table 4

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for corpus luteum (CL) subjective scores as a method of early pregnancy diagnosis at 21 d post-TAI based on a reference pregnancy diagnosis at 33 d post-TAI in Nelore heifers.

	Luteal Vascular Perfusion Score	CL Size Score	CL Echogenicity Score	CL Size Score + CL Echogenicity Score
Sensitivity	100% (235/235) ^a	96.6% (227/235) ^b	91.9% (216/235) ^b	93.2% (219/235) ^b
Specificity	79.1% (261/330) ^{ab}	76.1 (251/330) ^b	71.2% (248/330) ^b	84.2% (278/330) ^a
PPV	77.3% (235/304) ^{ab}	74.2 (227/306) ^{ab}	72.5% (216/298) ^b	80.8% (219/271) ^a
NPV	100% (261/261) ^a	96.9 (251/259) ^b	92.9% (248/267) ^c	94.6% (278/294) ^{bc}
Accuracy	87.8% (496/565) ^a	84.6 (478/565) ^{ab}	82.1% (464/565) ^b	88% (497/565) ^a

^{a-c}Percentages within a row with different letters differ ($P < 0.05$).

methods evaluated. Early pregnancy diagnosis using CL vascular perfusion score had the maximal (100%) sensitivity and NPV due to the absence of false negative diagnoses. The proportion of false negative diagnosis for CL size score, echogenicity score and CL size + echogenicity score were 3% (8/259), 7.1% (19/267), and 5.4% (16/294), respectively; resulting in a lesser ($P = 0.0002$) sensitivity and NPV than by using the CL vascular perfusion score for early pregnancy diagnosis. Percentage of false positive pregnancy diagnoses was greater ($P = 0.01$) when using the echogenicity score (27.5%; 82/298) compared to assessment using the CL size + echogenicity score (19.2%; 52/271). The rate of false positive diagnosis for CL size score (25.8%; 79/306) and luteal vascular perfusion score (22.7%; 69/304) was similar with the use of all the other methods. The accuracy of early pregnancy diagnostic method was greater for the luteal vascular perfusion score and CL size + echogenicity score than for the method only using the echogenicity score. The accuracy of using the CL size score for early pregnancy diagnosis was similar compared to all the other subjective methods (Table 4). The area under the curve was not different when using the luteal vascular perfusion score and CL size score methods of pregnancy diagnosis (Fig. 2).

4. Discussion

The present study was conducted to compare different methods of early pregnancy diagnosis in Nelore heifers at 21 d post-AI based on CL morphological characteristics as assessed by color Doppler and B-mode ultrasonography. Results of previous studies indicate that subjective evaluation of luteal vascular area using color Doppler, which is related to CL progesterone production capacity, could be used to identify non-pregnant cows between d 20 and 21 after AI with precise sensitivity (Pugliese et al., 2014; Scully et al., 2014). The evaluation of luteal blood flow using color Doppler, however, is a more complex examination that requires more time and personnel experience than the B-mode ultrasonography examination of ovaries. Besides, ultrasonic equipment with color Doppler capacity is more expensive than B-mode ultrasonic equipment (Pugliese et al., 2017), and replication of results of luteal blood flow in the field may be more complicated due to the varying machine settings and lack of technique standardization (Scully et al., 2015). Results of the present study indicate CL diameter had a similar sensitivity and negative predictive value, indicating that this may be an alternative approach to identify non-pregnant Nelore heifers at 21 d post-AI in beef cow-calf operations. Corpus luteum diameter, unlike the other objective CL morphological parameters (ColorPix and EchoPix), is measured easily and provide an immediate diagnostic response at the time of the examination, allowing the application of this technology extensively in the field. Furthermore, methods of early pregnancy diagnosis that provide for a rapid response permit an earlier enrollment of non-pregnant heifers and cows in estrous re-synchronization programs and a shorter inter-insemination interval.

It was hypothesized that an early pregnancy diagnosis at 21 d after TAI using CL vascularity evaluation assessed using color Doppler ultrasonography would result in a similar accuracy and sensitivity compared with use of the CL diameter and/or echogenicity evaluations using B-mode ultrasonography. Results of the present study indicate that the sensitivity and accuracy of early pregnancy diagnosis were not different when there was use of luteal vascularity and CL diameter for objective measurements, which partially supports the main hypothesis for the present research. With use of the objective echogenicity/luteal area (EchoPix) method, there is a lesser accuracy and greater proportion of false negatives. Furthermore, with use of the luteal echogenicity score and the combination of CL size and luteal echogenicity score, there was a greater proportion of false negatives compared to the use of the luteal vascular perfusion score. The early pregnancy diagnosis method using EchoPix had a lesser accuracy and smaller area under the curve compared with use of the CL diameter and ColorPix methods. This result was expected because in a previous study there was no effect of pregnancy status (pregnant and non-pregnant) and the day after AI (from 18–21 d) on CL echogenicity (Scully et al., 2014). The accuracy of the early pregnancy diagnosis using the echogenicity score was improved when used in combination with CL size score due to a reduction in the proportion of false positives. It, however, was not sufficient to avoid the occurrence of false negatives.

The subjectively morphological evaluation of ovarian structures is considered to be effective because of the correlation coefficient (r) being greater than 0.80 (de Tarso et al., 2016). There was a correlation ($r > 0.80$) between vascular perfusion and CL size scores with ColorPix and CL diameter, respectively, for all evaluators. Nevertheless, the correlation between luteal echogenicity score and EchoPix was less than desirable ($r < 0.70$) when there was assessment of results for all evaluators. It, therefore, appears that assessments using the luteal echogenicity method did not improve assessment effectiveness of CL functionality as compared with other luteal morphological characteristics, such as luteal area, CL diameter and CL vascular area (Silva and Ginther, 2010; Martins et al., 2011; Guimaraes et al., 2015).

In a recent review article, there was the conclusion that early diagnosis of pregnancy methods are considered to be effective when there is a 80%–95% accuracy, with absence or very low occurrence of false negative diagnoses (Pugliese et al., 2017). The absence of

false negative diagnoses is an important factor for early pregnancy diagnosis because cows identified as non-pregnant are commonly treated with luteolytic hormones in estrous timed-AI resynchronization programs that induce abortion such as prostaglandin F_{2α} (Fricke et al., 2016). In the present study, the use of CL subjective scores as assessed using B-mode or color Doppler for early pregnancy diagnosis were considered to have a satisfactory accuracy (> 80%). Only with use of the vascular perfusion score method, however, was there an absence of false negatives. The other CL subjective score methods had a range of 7.1%–3.1% of false negative diagnoses.

On the contrary, with use of the CL diameter and ColorPix objective methods, there was a satisfactory accuracy (86.7% and 89.4%, respectively) and an absence of false negatives. Guimaraes et al. (2015) also previously determined that with scores of luteal vascularity and CL size there was a satisfactory accuracy (88.3% and 82.8%, respectively) to diagnose pregnancy at d 21 of gestation (14 d after embryo transfer) in lactating dairy cows. In this previous study, with use of CL luteal vascularity score method there were zero false negatives, and with use of the CL size score method there was 5.6% false negatives (Guimaraes et al., 2015). In contrast to the present study and that of Guimaraes et al. (2015), the results from other studies indicated that with use of luteal vascular scores for early pregnancy diagnosis between d 20 and 21 after AI there were false negative diagnoses (1.5%: Siqueira et al., 2013; 2.5% Scully et al., 2014). This variation of results may be due to differences in machine settings, evaluation criterion, and/or technician experience to score luteal vascularity.

The percentage of false positives did not differ for all objective CL measurements methods of early pregnancy diagnosis, ranging from 20.3% to 24.2%. When analyzing subjective CL scores, the percentage of false positives ranged from 27.5% to 19.2%. The percentage of false positives for pregnancy diagnosis using the luteal echogenicity score method (27.5%) was less when analyzed in combination with CL size score (19.2%). Proportion of false positives when luteal blood perfusion score was used as an early pregnancy diagnosis method ranged from 19.6% to 35.2% in previous studies (Utt et al., 2009: 35.2%; Siqueira et al., 2013: 34.9%; Pugliesi et al., 2014: 19.6%; Scully et al., 2014: 21.2%; Guimaraes et al., 2015: 21.1%). The large variation in proportion of false positives reported in these previous studies may be due to (1) differences in machine settings, (2) variation of evaluation criterion, (3) distinction of evaluator experience to score luteal vascularity, (4) insemination of cows and heifers not in estrus or not estrous synchronized, (5) cows and heifers with luteolysis after the early pregnancy diagnosis, and (6) cows and heifers with pregnancy loss between early and late times of pregnancy diagnosis (Scully et al., 2014; Diskin et al., 2016; Wiltbank et al., 2016; Pugliesi et al., 2017, 2018). Cattle breeds, parity, herds, season, and location may also affect those factors and may also affect the accuracy when there is use of early pregnancy diagnosis methods utilizing parameters of CL functionality. In the present study, heifers with a CL diameter of > 13 mm were considered to have a functional CL and be pregnant based on the early pregnancy diagnosis. In a previous study, only Holstein cows with CL diameter of > 23 mm were considered to have a functional CL at 21 d after AI (Bicalho et al., 2008). The criterion used for early pregnancy diagnosis in the present study was, therefore, specific for grazing nulliparous Nelore heifers and may not be adequate for assessment of early pregnancy in other cattle types such as multiparous high producing lactating Holstein cows.

In summary, CL diameter, as assessed using B-mode ultrasonography had a similar accuracy and proportion of false diagnoses when used for early pregnancy assessment as compared with the evaluation of the vascular perfusion method using color Doppler ultrasonography in grazing nulliparous Nelore heifers. Measuring CL diameter may be a simpler, less expensive, and more rapid method to identify non-pregnant Nelore heifers at 21 d post-TAI. Early pregnancy diagnosis using the subjective scores of CL morphology as assessed using B-mode ultrasonography was of a similar accuracy compared to assessments using the scores of CL vascular perfusion obtained using color Doppler. The occurrence of false negative pregnancy diagnoses using only the CL morphology scores may make this method of early pregnancy diagnosis unfeasible.

Declaration of Competing Interest

The authors declare no conflict of interest.

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