



Original Research Article



Identification of nonpregnant beef cows based on CL size vs. luteal blood perfusion at 21 days after timed artificial insemination

João Paulo N. Andrade^a, Rafael R. Domingues^{a,b}, Pedro L.J. Monteiro^{a,c}, José Ricardo Dias^d, Cesar Pimenta^d, Afonso S. Guimarães^e, Leuton Barbosa^f, Sami Merhi^d, Roberto Sartori^e, Milo C. Wiltbank^{a,*}

^a Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Wisconsin, 53706, USA

^b Department of Animal Sciences, The Ohio State University, Columbus, OH, 43210, USA

^c Department of Large Animal Clinical Sciences, University of Florida, Gainesville, FL 32608, USA

^d Agropecuária Roncador, Av. Rio Grande do Sul, 387, Querência, MT, 78643-000, Brazil

^e Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Av. Pádua Dias, 11, Piracicaba, SP, 13418-900, Brazil

^f Agrobef Ltd, Avenida Senador Valdon Varjão, 4025, Barra do Garças, MT, 78605-970, Brazil

ARTICLE INFO

Keywords:

Bos indicus
Reproductive efficiency
Resynchronization
Corpus luteum
Ultrasonography

ABSTRACT

The aim was to evaluate the efficiency of two different ultrasonographic systems, Doppler mode vs. Two-dimensional mode (B mode), to identify the pregnancy status of beef cows and heifers on day 21 (D21) after Timed Artificial Insemination (TAI). The experiment was performed on a commercial beef farm in central-west region of Brazil using 1895 Nelore heifers and cows. All females had ovulation synchronized for a TAI that was performed on D0. Twenty-one days after the TAI, all animals had their ovaries evaluated by ultrasound for pregnancy diagnosis based on the size of the corpus luteum (CL). Using B mode ultrasonography, females without a CL or with a CL ≤ 10 mm in diameter were considered nonpregnant, whereas females with a CL > 10 mm in diameter were considered potentially pregnant. After the B mode examination, the Doppler mode was turned on, and the CL was evaluated by the subjective percentage of blood perfusion in the total area of the CL. Using Doppler mode, females were considered nonpregnant if they had no CL or the CL had ≤ 25 % of the total area with detectable blood perfusion, whereas animals with > 25 % blood perfusion in the CL were considered potentially pregnant. The results for each method (potentially pregnant or nonpregnant) were later compared with the gold standard technique, which was a pregnancy diagnosis on D33 after TAI using ultrasound with visualization of an embryonic heartbeat. The accuracy was determined using the 2×2 contingency table approach. The area under the curve using the receiver operating characteristic curve for Doppler mode and B mode were 0.929 and 0.902 ($P < 0.01$), respectively. There were almost no false negatives (designated nonpregnant but later pregnant at D33) with either technique (0.2 % vs. 0.3 %; $P = 0.65$ for Doppler mode vs. B mode, respectively). False positives (designated pregnant but non-pregnant on D33) were greater for B mode compared to Doppler (19.1 % vs. 14.0 %; $P < 0.01$). This resulted in Doppler mode having similar high values as B mode for Negative Predictive Value (99.9 vs. 99.6 %; $P = 0.85$) and Sensitivity (99.8 vs. 99.7 %; $P = 0.86$) but there were differences in Specificity (86 vs. 80.9 %; $P < 0.01$), Positive Predictive Value (88 vs. 84.3 %; $P < 0.01$), and Accuracy (93.0 vs. 90.4 %; $P < 0.01$). In conclusion, evaluation of CL blood perfusion by Doppler produced greater accuracy in the early identification of nonpregnant heifers and cows on D21 after TAI than measurement of CL diameter with B mode ultrasound; although both had over 90 % accuracy in identifying pregnant and nonpregnant females.

1. Introduction

Use of rectal palpation for pregnancy diagnosis after the end of the

breeding season with subsequent culling of non-pregnant cows was one of the first strategies used to improve the economics and reproductive performance of beef cattle operations. Veterinarians could perform a

* Corresponding author.

E-mail address: wiltbank@wisc.edu (M.C. Wiltbank).

<https://doi.org/10.1016/j.theriogenology.2024.08.014>

Received 27 November 2023; Received in revised form 9 August 2024; Accepted 12 August 2024

Available online 23 August 2024

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pregnancy diagnosis using rectal palpation with high accuracy (>95 %); however, only after ~40 days in gestation [1]. As new reproductive biotechniques, such as Timed Artificial Insemination (TAI), were incorporated into cow-calf operations, it became desirable to have earlier identification of pregnancy status in order to have more intensive programs that produced greater reproductive efficiency [2]. Rectal ultrasonography became the gold standard technique to determine pregnancy status with high accuracy at ~30 days after TAI [3]. The ultrasound in the two-dimensional mode (B mode) provides visualization of the embryo so that pregnancy diagnosis is determined by direct detection of an embryo with a positive heartbeat and selection of appropriate approaches for rebreeding any nonpregnant cows during the breeding season [4–6]. Nevertheless, to further optimize reproductive efficiency, nonpregnant cows should ideally be bred before D30 after the previous TAI, which is earlier than typically done using classical pregnancy diagnosis with B mode ultrasonography. Therefore, indirect methods for earlier pregnancy diagnosis emerged as a possibility to develop reproductive programs with shorter interbreeding intervals.

Indirect methods for pregnancy diagnosis have utilized laboratory assays that target the presence or absence of molecules (hormones, proteins, or mRNA) that are associated with a specific stage of the female reproductive cycle and/or the pregnancy status of the female with some degree of accuracy [7–9]. The accuracy of each technique relies on its ability to precisely detect pregnant (specificity) or nonpregnant (sensitivity) cows [10]. For indirect methods of pregnancy diagnosis, false negative results must be avoided as this misdiagnosis may lead to pregnancy loss in pregnant cows incorrectly treated with prostaglandin (PG) F₂α. On the other hand, high incidence of false positives decreases the utilization rate of nonpregnant cows in the subsequent TAI program [11]. The most common molecules that are used for pregnancy diagnosis in cattle are progesterone (P4; in blood or milk samples), pregnancy-associated glycoproteins (PAGs; in blood or milk samples), and mRNA for interferon-stimulated genes (ISGs; in cells from blood, vagina and/or cervix) [12–17]. These techniques require collection of a biological sample (blood, milk, or vaginal/cervical epithelial cells) and processing/analyzing either in a laboratory or at the farm. In practical terms, however, immediate results (such as in ultrasonography or rectal palpation) are critical in beef cattle operations to optimize decision-making strategies that minimize animal handling and reduce intervals between breeding. Thus, although P4 tests are generally impractical on commercial beef cattle operations, identification of cows with high P4 can be immediately determined indirectly by assessing corpus luteum (CL) blood perfusion using color Doppler ultrasound (Doppler mode) [18]. This has recently been used as an indirect method for early pregnancy diagnosis in cattle [16,19–21].

Early reports showed dramatic changes in ovarian and uterine blood perfusion during the reproductive cycle and pregnancy of cattle using Doppler ultrasound [22,23] and strategies were proposed to use CL blood perfusion as a technique for early pregnancy diagnosis [21,24]. To produce a practical tool, researchers proposed a classification system for luteal blood perfusion using subjective scores of the total CL area containing blood perfusion, reporting a similar or better accuracy compared to circulating P4 or ISGs in beef cattle [16,22–25]. Using this technique for early pregnancy diagnosis, novel reproductive programs were proposed that allowed resynchronization of ovulation and shorter intervals between breeding of 24, 23, or even 21 days between TAIs [21,24,26,27]. Importantly, there were few or no false negative diagnoses reported when using the Doppler mode as a technique for earlier nonpregnancy diagnosis. However, assessing CL blood perfusion with Doppler mode requires technical skill under field conditions to accurately classify cows as pregnant or nonpregnant. In addition, ultrasound machines equipped with Doppler are more expensive than a B mode ultrasound, again making them less practical in the field. Interestingly, research prior to the studies exploring Doppler for pregnancy diagnosis, evaluated the use of CL size by B mode ultrasound for early identification of nonpregnant dairy cows but the researchers reported low accuracy, discouraging

further development of the technique [28]. Nevertheless, our recent study and a previous study from another laboratory comparing CL ultrasonography images using Doppler mode vs. B mode in Nelore heifers or cows showed similar accuracy, but a limited number of females were used in both these experiments (n = 113 [29]; n = 110 [16]).

Thus, the present study was designed to compare the use of Doppler-mode (CL blood perfusion) vs. B-mode (CL size) to determine the precision of these techniques for early nonpregnancy diagnosis on D21 in a large cow/calf operation. We hypothesized that both techniques would have: (1) similar accuracy, (2) similar precision to detect pregnant cows (specificity and positive predictive value), and (3) similar precision to detect nonpregnant cows (sensitivity and negative predictive value).

2. Materials and methods

The experiments were performed at Roncador Farm, located in Querência, MT, Brazil. Animals were kept on pasture (*Brachiaria brizantha*) supplemented with mineral salt and had ad libitum access to water. Nelore *Bos-indicus* beef cattle (n = 1895) from different parities (Nulliparous [n = 836] kept in two different pastures, Primiparous [n = 534] kept in three different pastures, and Multiparous [n = 525] kept in three different pastures) were used in the study. The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures. Heifers/cows were enrolled in an estradiol/P4-based protocol that allowed first TAI (designated D0) [30]. The treatments began when multiparous and primiparous cows were approximately 35d after calving, and heifers were at 14–20 months of age with ≥280 kg of body weight. All TAI were performed by five experienced technicians using frozen/thawed semen from sires of proven fertility (five Rubia Gallega or two Nelore).

A commercial ultrasound machine (SonoScape E2 PRO) B mode (6.8–10.1 MHz frequency probe, settings: 8 cm depth, 170 gain, and 140 dynamic range) and color Doppler-mode (5.7 MHz frequency, settings: 80 color gain, 130 gain, 130 color filter, and 1.2 kHz pulse repetition frequency) was used for ultrasonography exams. All the ultrasound exams were performed by one experienced technician. The B mode evaluation of ovaries utilized subjective structural features of the CL. To aid in estimating the CL size, images were compared to two vertical white lines that were fixed on the ultrasound screen at 10 mm apart (Fig. 1).

On D21 (21 days after TAI), the B mode ultrasonography of the CL was performed with Doppler mode off. Heifers/cows were classified into

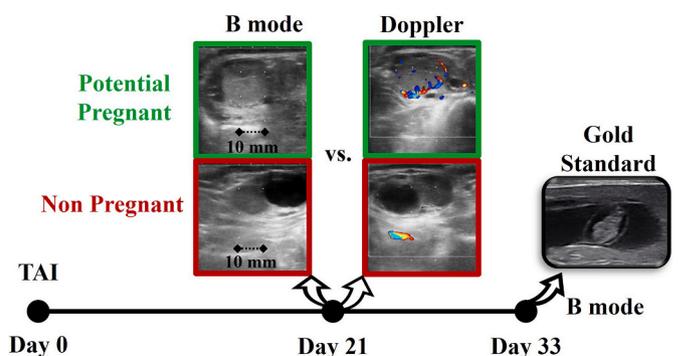


Fig. 1. Illustration of the experimental design. On D21 after TAI the early pregnancy diagnosis was performed using B mode ultrasound based on estimation of CL size using as a reference two vertical lines fixed in ultrasound screen 10 mm apart from each other. After CL size score estimation, the Doppler mode was turned on for estimation of CL blood perfusion based on subjective scores. All results were compared to the gold standard method of B mode ultrasound on D33 by visualization of embryonic heartbeat. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

two subjective categories and information on B mode was recorded for later analysis: nonpregnant (no CL or CL ≤ 10 mm of diameter) and potentially pregnant (at least one CL > 10 mm of diameter). After the ovaries were examined by B mode, the Doppler mode was then turned on, and CL were evaluated using subjective scores of blood perfusion [29] and classified into two categories: nonpregnant (no CL or CL with ≤25 % of the total area with color blood perfusion signals) and potentially pregnant (at least one CL with >25 % of the total area with color blood perfusion signals).

The gold standard diagnosis for pregnancy was the ultrasonography exam on D33 after TAI. Heifers/cows with a positive embryonic heart-beat were considered pregnant. False positives were cows/heifers that were classified as potentially pregnant by the early nonpregnancy diagnosis (on D21) but were not pregnant on D33 divided by the number of all nonpregnant heifers/cows on D33. False negatives were heifers/cows that were nonpregnant by the early pregnancy diagnosis (on D21) but were pregnant on D33 divided by the number of all pregnant females on D33.

2.1. Statistical analyses

The precision of each method was evaluated using the 2 × 2 contingency table approach [29]. Data were analyzed with SAS (Version 9.4; SAS Institute) using PROC GLIMMIX for binomial data (negative predictive value [NPV], positive predictive value [PPV], specificity, sensitivity, accuracy, false negative, and false positive). The statistical model includes as fixed effects Sire, Parity and US mode as a last predictor to estimate extra sum of squares from US mode. Each pasture has unique parity distribution, thus pasture was not included in overall model to avoid multicollinearity when two or more explanatory variables are moderately or highly correlated. But, within parity analysis for false positive Pasture was include in the model instead of Parity. Receiver operating characteristics analysis was performed using PROC LOGISTIC. Significant differences between techniques were considered for P ≤ 0.05, whereas differences between P > 0.05 and P ≤ 0.10 were considered a tendency.

3. Results

Table 1 shows the frequency of different diagnoses in *Bos indicus* Nelore heifers and cows on D21 after TAI using ultrasound on either B mode to evaluate CL size vs. Doppler mode to evaluate CL blood perfusion. The true positive rate was 86.0 % for Doppler and 80.9 % for B mode with an effect of ultrasound mode (P < 0.01) and Parity (P < 0.0001). Accordingly, false positives were greater in B mode than Doppler (P < 0.01) and greater for heifers than for primiparous or multiparous (P < 0.0001) cows (Table 1). In contrast, there were very few false negatives in either group (≤0.3 %) with no differences between ultrasound method (P = 0.65) resulting in true negatives in both groups

Table 1

Incidence of different true (correct) and false (incorrect) diagnoses for an early pregnancy diagnosis using CL morphology based on B-mode ultrasound or CL blood perfusion based on Doppler ultrasound.

	Ultrasound mode		P-value		
	Doppler	B mode	Mode	Parity	Mode*Parity
True Positives (930)	86.0 % (800)	80.9 % (752)	<0.01		
True Negatives (965)	99.8 % (963)	99.7 % (962)	0.99		
True Diagnoses (1,895)	93.0 % (1763)	90.4 % (1714)	<0.01		
False Positives (930)	14 % (130) a	19.1 % (178) b	<0.01	<0.0001	0.89
Heifers (483) a	16.1 % (78)	21.3 % (103)	0.05		
Primiparous (231) b	10.4 % (24)	16.0 % (37)	0.07		
Multiparous (216) b	13.0 % (28)	17.6 % (38)	0.17		
False Negatives (965)	0.2 % (2)	0.3 % (3)	0.65		

The false-positive rate was calculated based on the total number of non-pregnant cows at day 33 of gestation, and the false negative rate was calculated based on pregnant cows at day 33 of gestation. Lowercase different letters indicate P < 0.01.

A. Receiver operating characteristic

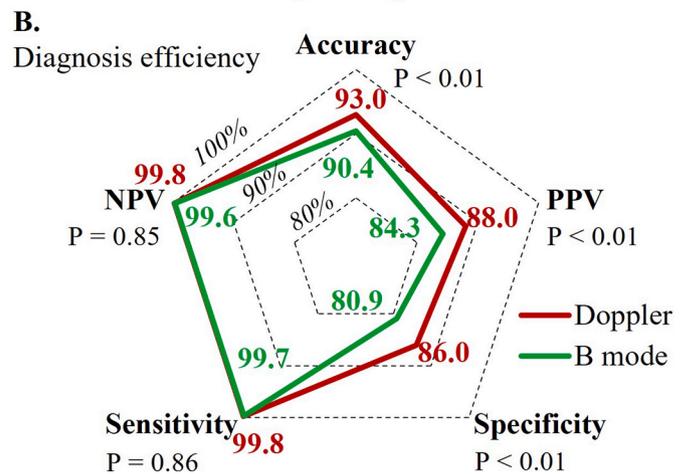
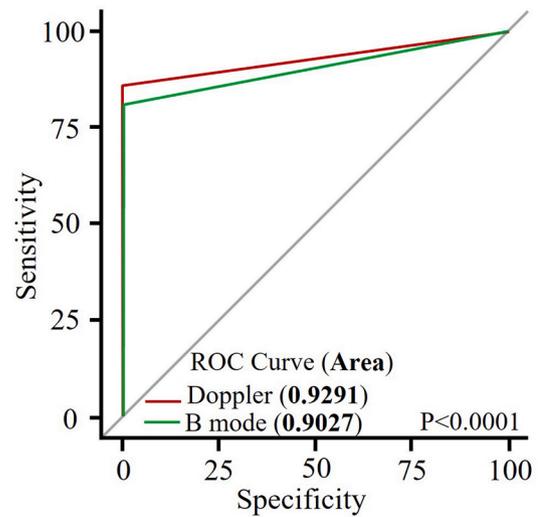


Fig. 2. Panel A. Receiver operating characteristics comparing the area under the curve for early methods of pregnancy diagnosis by B mode vs. Doppler ultrasound. Assessing the differences in sensitivity and specificity generates a curve; an area under the curve closest to 1 means more accuracy in the test. Panel B. Accuracy, Sensitivity, Specificity, NPV (negative predictive value), and PPV (positive predictive value) obtained by 2 × 2 contingency approach analysis. The dashed line in the pentagon indicates 80 %–100 %. Individual values of each test are indicated; values closest to 100 % indicate more precision for each test.

of >99 %. Since the incidence of false negatives was almost zero in both groups, these data were not analyzed by parity. The overall accuracy or true diagnoses were greater ($P < 0.01$) for Doppler at 93.0 % (1763/1895) than for B mode 90.4 % (1714/1895).

Receiver operating characteristics (ROC) and efficiency of Doppler mode and B mode ultrasonography for early identification of non-pregnancy status of beef cows/heifers are shown in Fig. 2. For the ROC curve analysis, Doppler mode had a greater area under the curve compared to B mode (0.9291 vs. 0.9027; $P < 0.0001$). The overall accuracy of pregnancy/nonpregnancy diagnoses performed by either Doppler or B mode ultrasound was greater than 90 %; yet Doppler mode was slightly (93.0 vs 90.4 %; 2.6 % absolute difference or 2.9 % relative difference) but significantly ($P < 0.01$) more accurate than B mode ultrasonography (Fig. 2B).

Since the frequency of false negatives was so low for both groups, both techniques were similar and had NPV and sensitivity above 99 %, indicating that both techniques were excellent in not classifying pregnant females as nonpregnant (Fig. 2). On the other hand, the PPV and specificity were different ($P < 0.01$) between techniques showing that B-mode designated more of the non-pregnant cows as pregnant compared to Doppler. For example, the PPV for Doppler was 88.0 % compared to only 84.3 % with B mode (3.7 % absolute difference and 4.2 % relative difference) ($P < 0.01$; Fig. 2B).

4. Discussion

Although methods of early pregnancy/nonpregnancy diagnoses have been previously compared [14,17,31], including comparisons of B mode vs. Doppler [16,29,32], the present study did these important comparisons in a commercial operation, under field conditions with a large number of females. Using the B mode ultrasound for subjective estimation of the CL size produced almost no false negatives, similar to the Doppler method using estimation of CL blood perfusion. Nevertheless, in contrast to previous studies [16,29] with limited numbers of females in the study, the present study using almost 2000 females detected a small (about 3 %) but significant increase in accuracy along with a lower incidence of false positives utilizing the Doppler mode compared to B mode [29]. Yet, both methods gave an overall accuracy above 90 %, showing that either method can be reliably used for nonpregnancy diagnoses at D21 after breeding. Importantly, our results demonstrate that these methods can be used to support current resynchronization practices by identifying nonpregnant heifers/cows under field conditions. Combining these early pregnancy diagnosis techniques with a rapid resynchronization program, such as ReBreed21 [33,34], can decrease the interval between TAI, potentially decreasing time to pregnancy and thereby increasing reproductive and productive efficiency on beef cattle operations. In this discussion we will focus on the factors affecting accuracy of the two methods, particularly the physiology behind the incidence of false positives and how these methods can be practically applied in beef cattle reproductive programs.

The overall accuracy of any diagnostic test is calculated using a gold standard which, in this trial, was direct ultrasound of the embryo on D33 after TAI including detection of an embryonic heartbeat. In contrast to our first hypothesis and to previous studies [16,29], the accuracy differed between these two techniques due to greater percentage of false positives with the B mode than with Doppler but both had an accuracy >90 %, which is generally considered acceptable [10].

For an earlier pregnancy diagnosis in cattle, the error that must be avoided is the false negative as this may lead to treatment with PGF2 α and an iatrogenic pregnancy loss [35]. In other studies and reproductive programs, nonpregnant heifers/cows received treatment with an analog of PGF2 α that could cause loss of the pregnancy [24,26,36]. Therefore, most early resynchronization programs would not have false negatives [35]. In this study no PGF2 α was given, as is typical for ReBreed21 [33], to nonpregnant females and both techniques (B mode and Doppler) showed a low incidence of false negative diagnoses. By comparison,

measurement of mRNA for ISGs in peripheral blood can have 10–30 % false negatives, limiting this technique to mainly research purposes [16,25,37]. Recently, measurement of ISGs in cervical or vaginal epithelial cells improves accuracy (Domingues et al., 2023) but the costs and need for a complex laboratory analysis still limits the usefulness of ISGs in commercial operations. The less complex pregnancy assays for circulating PAGs have a low incidence of false negatives when used after D27 [14,15,17,38]. A recent study in lactating dairy cows showed that an increase of at least 10 % in optical density from D17 to D24 after TAI had 100 % sensitivity, although this approach would likely not be practical on beef cattle operations due to the requirement to collect multiple samples [39]. Thus, based on infrequent false negatives, measurement of either CL diameter or CL blood perfusion can be effectively used for nonpregnancy diagnosis, although under field conditions there are a few false negatives, probably due to technical issues such as miscommunication during the ultrasound exam. These rare mistakes highlight the importance of avoiding treatment with a PGF2 α after early pregnancy diagnosis either by Doppler or B mode ultrasound.

The other side of accuracy for an early pregnancy diagnosis technique is the positive predictive ability of the method. Our second hypothesis, based on previous results [16,29], was that B mode and Doppler mode would have similar precision in detecting pregnant cows; although we knew that false positive diagnoses have been frequently reported with any technique, including CL blood perfusion, used near D21 of bovine pregnancy [21,24]. Our results did not support our second hypothesis since the false positives were greater using B mode than Doppler in all parities. Thus, about 5.1 % of cows that were diagnosed nonpregnant based on CL blood perfusion were diagnosed pregnant by CL size using B mode ultrasound. This difference is likely due to differences in the timing among measurable CL functions. For example, a 50 % decrease in circulating P4 precedes the 50 % decrease in CL blood perfusion by about 0.5 days, and this is followed by the decrease in CL diameter 0.5 to 1d later [40–42]. That is, during luteolysis, CL blood perfusion decreases earlier than the decrease in CL size. Therefore, the greater false positives based on CL size are likely due to heifers/cows that are in the earlier stages of luteolysis when CL blood perfusion had decreased but there was not yet a large enough change in CL size to be designated nonpregnant [43,44]. Nevertheless, there were also a substantial number of females that were false positives, even with the Doppler technique. These are likely due to either early pregnancy loss between D21 and D33 or later CL regression in nonpregnant females. A high incidence of pregnancy loss between D21 and D33 has been reported in dairy cows [37,45,46] but this is likely lower in beef cattle than dairy cattle [24,26,36]. Several studies have labelled false positives as pregnancy losses [24,26]. However, nonpregnant females that have CL regression later than 21 days would also be false positives. In other studies using nonbred Nelore heifers and cows some animals had luteolysis later than D21 [47], for example, we observed 22.2 % (12/54) of nonbred Nelore heifers had luteolysis after D21, suggesting that many false positives may be due to later luteolysis [48]. Of special interest, heifers had greater incidence of false positives than cows perhaps due to a combination of greater pregnancy losses, as would be expected [36], and greater incidence of four follicular wave patterns (luteolysis after D21). Thus, false positives are a problem with either method of early pregnancy diagnosis and this study has provided a reliable estimate of this problem in *Bos indicus* cattle using the two ultrasound methods and evaluating almost 2000 cows/heifers.

Finally, the practical role for a method that precisely identifies nonpregnant females at earlier stages of pregnancy is primarily related to potential incorporation in a reproductive program that includes a rapid rebreeding programs [10]. The use of ultrasonography either by Doppler mode or B mode conveniently provides the information in real time at cow side, thus providing on-farm applicability. For example, combining ReBreed21 with either ultrasound technique would allow 80 % or more of the nonpregnant cows that would be identified on D33, to be bred at D21 after previous TAI permitting completion of an entire

breeding season with three potential TAIs in 42 days. This strategy can be particularly effective in heifers due to the inherent lower fertility at first AI and relatively high fertility to the ReBreed21 program [34]. Several studies have reported that a shorter, more intensive breeding season with shorter postpartum anestrus can increase herd reproductive performance consequently increasing the profitability of the operation by producing more calves and heavier calves at weaning and producing more fertile replacement heifers [49–51]. An important practical finding of this large study was that either B mode or Doppler can be used with low risk for iatrogenic pregnancy losses, due to their exceptionally low frequency of false negatives, using the 10 mm diameter criteria applied in this study. On the other hand, using B mode produced greater false positives than Doppler, thus decreasing the number of nonpregnant cows receiving a new earlier TAI. Of importance, there are several limitations for our study. First, only a single very conservative cutoff was utilized to determine non-pregnancy. Other studies that have utilized a larger CL size (CL area <2 cm²; ~16 mm diameter) report a much greater number of false negative results [32]. Second, future practical economic studies are needed to model and investigate the investments/returns using different lengths of breeding season, methods of early pregnancy diagnosis, and rebreeding strategies, particularly comparing whether the economics differ by parity. These studies should focus on one of the prime endpoints for profitability in beef cattle, the weight of calves at weaning. Some studies have used simulated computer models to predict the production and economic performance of beef cattle herds under different reproductive management programs (10 different scenarios) that included resynchronization programs that used Doppler ultrasonography to determine pregnancy [50,51]. Use of TAI and use of more rapid resynchronization programs (24 days between AIs in their most aggressive scenario) were associated with weaning of more calves and heavier calves [51] and generally better economics than use of natural mating or less aggressive programs [50]. Nevertheless, further studies are needed that directly compare programs in beef cattle herds, rather than just with computer simulations, to validate the concept that more aggressive TAI programs using rapid resynchronization programs, such as ReBreed21, can improve production and economics of commercial beef cattle operations. A third limitation of our study is that a single, well-experienced ultrasound technician performed all examinations in this study and studies are needed to compare the variability in results obtained with other ultrasound technicians under field conditions.

5. Conclusion

In summary, using B mode ultrasonography as an early (D21) pregnancy diagnosis procedure in comparison to Doppler mode ultrasound had similar and low incidence of false negatives but greater number of false positives, resulting in lower accuracy. Hence, the present results demonstrate that B mode can be used as a fairly accurate method to select nonpregnant heifers/cows at an earlier stage of pregnancy (D21) but the slight but significant increase in false positives is likely to reduce efficiency of the rebreeding program. Future studies should evaluate the economics and efficiency of using these two techniques within practical rebreeding programs during a realistic breeding season on commercial beef cattle operations.

CRedit authorship contribution statement

João Paulo N. Andrade: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rafael R. Domingues:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Pedro L.J. Monteiro:** Writing – review & editing, Formal analysis. **José Ricardo Dias:** Methodology, Investigation. **Cesar Pimenta:** Methodology, Investigation. **Afonso S. Guimarães:**

Methodology, Investigation, Data curation. **Leuton Barbosa:** Supervision, Resources, Methodology, Investigation. **Sami Merhi:** Methodology, Investigation. **Roberto Sartori:** Writing – review & editing, Resources, Project administration, Formal analysis. **Milo C. Wiltbank:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Acknowledgments

The authors thank Alexandre B. Prata, Adelino J. Robl, José Neto, Beatriz Lipped, Heuller S. Ribeiro, Diego Hartman, the owners, and staff of the farm Roncador (Mato Grosso state) for their contribution with the heifers and facilities. Support was provided by USDA Hatch Project WIS01240 to MCW and USDA-NIFA project 2019-67015-29704 to MCW.

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