Using pregnancy-associated glycoproteins to provide early pregnancy diagnosis in Nelore cows

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ABSTRACT

Pregnancy-associated glycoproteins (PAG) are expressed in the placenta of ungulate mammals. Ruminants secrete PAGs continuously from embryonic implantation until delivery. In this study, we evaluated the feasibility of using ELISA to detect PAG levels as a method for early pregnancy diagnosis in Zebu (Nelore) beef cows. We compared the efficacy of this method with transrectal ultrasonography (US) in detecting pregnancy on the 25th and 28th days after timed artificial insemination (TAI) of 130 crossbred Zebu cows. Blood samples were taken during these two time points for PAG determination; ultrasound examinations were also performed on both days. Rectal palpation was used on the 80th day to definitively verify pregnancy. PAG ELISA accuracy was equal across both time points, with 100% sensitivity, 92.86% specificity, 96.70% positive predictive value, and 100% negative predictive value. In contrast, US had 62.50% and 93.18% sensitivity, 95.24% specificity, 96.49% and 97.63% positive predictive value, coupled with 54.79% and 86.96% negative predictive value on the 25th and 28th days, respectively. Thus, the two diagnosis methods differed significantly in negative predictive value and sensitivity, but not positive predictive value or specificity. In conclusion, PAG ELISA was just as specific as US, but more sensitive. Its safety (compared with US and rectal palpation) and high accuracy in identifying non-pregnant cows make PAG ELISA a good option for early pregnancy diagnosis (beginning 25 days post-TAI) in Zebu females.

1. Introduction

Early pregnancy detection is useful for increasing cattle reproductive efficiency. Non-pregnant cows can be identified quickly after the final service rates, minimizing economic losses that occur if pregnant cows are erroneously culled or non-pregnant cows are retained. Furthermore, service rates can be indirectly increased through lower birth-conception or between-birth intervals (Green et al., 2011; Ribeiro et al., 2012).

For optimal identification of early pregnancies, were require effective diagnostic methods that are accurate, practical, rapid, and cost-

effective (Karen et al., 2015). To that end, an increasingly popular technique for diagnosing gestation is quantifying pregnancy-associated glycoproteins (PAG), inactive aspartic proteases in the trophoblasts of placental binucleated cells. From day 24 post-fertilization, PAGs are released into the maternal bloodstream, becoming an important marker of pregnancy and fetal viability (Wallace et al., 2015). The use of PAG quantification were positively correlated with normal pregnancies being important indicator of fetal viability (Dobson et al., 1993).

Existing PAG analyses of dairy and beef cattle (Ricci et al., 2015; Roberts et al., 2015) have typically focused on taurine cows (*Bos taurus taurus*) (Commun et al., 2016; Shahin et al., 2014). These studies report

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that *B. taurus indicus* have higher PAG concentration during gestation (between 32 and 62 days post-insemination) than *B. taurus taurus*, suggesting a genetic influence on protein profiles. Thus, such techniques for gestational diagnoses must be specifically validated in Zebu breeds to enhance their effectiveness and broaden their applicability (Mercadante et al., 2013).

With this objective in mind, here we evaluated the viability of a commercial ELISA kit in detecting PAG concentrations of Nelore cows at 25 and 28 days post-insemination. We also aimed to compare the effectiveness of the PAG ELISA technique with standard methods for early pregnancy diagnosis, including ultrasound and transrectal palpation.

2. Materials and methods

2.1. Study site

The present study was conducted on a farm located in the municipality of São Francisco do Pará (located at kilometer 14 of the PA-320 highway - 01°10'15" South, 47°47'38" West), in the Brazilian state of Pará, Amazonia.

Subject females were raised with the other animals of the farm during their reproductive phase. All animals were fed exclusively in cultivated pastures of *Urochloa humidicola*, *U. brizanta* 'Marandu', and *Megathyrus maximus*, with *ad libitum* mineral supplementation and water. Animals were vermifugated soon after birth, then subsequently in 6-month intervals, concomitant with vaccinations against foot-and-mouth disease, symptomatic carbuncle, rabies, and brucellosis.

2.2. Experimental set-up

A three-management timed artificial insemination (TAI) protocol was applied to 130 healthy Zebu Nelore females (*B. taurus indicus*) with a mean body condition score of 3.5 (scale of 1 to 5) (Ayres et al., 2009). On day 0, one gram of progesterone (DIB®, MSD Saúde Animal, São Paulo, Brazil) was delivered using an intravaginal device for 8d. Additionally, still on day 0, a 2.0 mg intramuscular injection of estradiol benzoate (EB) (Estrogin®, Farmavet, São Paulo, Brazil) was applied. On the afternoon of day 8, subjects were treated a second time with the same dosage of EB (1.0 mg im), along with 0.15 mg D-cloprostenol (Preloban®, Intervet, Boxmeer, Netherlands) and 300 IU of equine chorionic gonadotrophin (Folligon®, MSD Saúde Animal, São Paulo, Brazil). Cows were artificially inseminated in the morning of day 10 (Sá Filho et al., 2011).

All females subjected to TAI protocol had a postpartum period of at least 35 days.

2.3. Determination of PAG concentrations

Blood samples (10 mL) were collected from all subjects on days 25 and 28 post-TAI, via puncturing the external jugular vein and using siliconized vacutainer tubes without anticoagulant. Samples were centrifuged for 15 min at 1150 g. The supernatant was stored in cryotubes at -20 °C until needed for PAG determination.

Pregnancy-associated glycoprotein concentration in bovine serum was determined following manufacturer protocol from the "Bovine Pregnancy Test" ELISA kit (IDEXX Laboratories, Westbrook, Maine, USA). Analyses were performed in the Virology Laboratory of the Environment Section at Evandro Chagas Institute.

2.4. Transrectal ultrasonography (US)

Ultrasound (US) examinations were performed on days 25 and 28 post-TAI, using a 7.5 MHz linear array transducer (Mindray DP 6900, Shenzhen, China). The observation of allantoic fluid or a visible fetus yielded a positive gestation diagnosis (Szenci et al., 1995). The same

veterinarian conducted all US evaluations.

2.5. Transrectal palpation

Transrectal palpation was the method chosen as the gold standard for subsequent confirmation of pregnancy. All cows were palpated at 80 days post-TAI by an experienced technician.

2.6. Statistical analysis

Accuracy is defined as the proportion of pregnant and nonpregnant cows correctly identified by the test [(true positive + true negative)/total population]. Results were classified as correct positive diagnosis (a), incorrect positive diagnosis (b), correct negative diagnosis (c), and incorrect negative diagnosis (d). Following published methods (Karen et al., 2011), sensitivity ($100 \times a / a + d$), specificity ($100 \times c / c + b$), positive predictive value (PPV) ($100 \times a / a + b$), and negative predictive value (NPV) ($100 \times c / c + d$) were calculated for each diagnostic method (PAG ELISA, US, palpation). A chi-square test determined significant differences in sensitivity/specificity between PAG ELISA and US. Differences between pregnant and non-pregnant cows determined via PAG ELISA were analyzed with the Student's *t*-test. Significance was set to $P < 0.05$.

3. Results

Rectal palpation 80 days post-TAI indicated that 67.69% (88/130) of subject cows were pregnant and 32.31% (42/130) were not. As shown in Table 1, the PAG ELISA test yielded three false positives, while US yielded two. No false negatives occurred under the PAG ELISA test. In contrast, considering only pregnant animals (88 cows), US identified as non-pregnant at 25 and 28 days post-TAI, respectively, 37.5% (33/88) and 6.8% (6/88).

The PAG ELISA test was equally accurate at 25 and 28 days post-TAI, reaching 100% sensitivity and 92.86% specificity. Ultrasound pregnancy detection was more accurate at 28 days post-TAI (sensitivity = 93.18% and specificity = 95.24%) than at 25 days (sensitivity = 62.50% and specificity = 95.24%) (Table 1).

The two tests did not significantly differ in specificity and PPV. However, PAG ELISA reached 100% negative predictive value at both evaluated time points, whereas US values were lower (day 25–54.79%; day 28–86.96%).

Table 1

Comparison of the results of the early diagnosis of pregnancy in Zebu cows using the PAG ELISA and transrectal ultrasonography (US) methods in relation to transrectal palpation.

Variables	25 days		28 days		80 days Transrectal Palpation
	PAG	US	PAG	US	
Correct positive results	88	55	88	82	88
False positives	3	2	3	2	0
Correct negative results	39	40	39	40	42
False negatives	0	33	0	6	0
Sensitivity, %	100 ^{Aa}	62.50 ^{Bb}	100 ^{Aa}	93.18 ^{Ab}	–
Specificity, %	92.86	95.24	92.86	95.24	–
PPV, %	96.70	96.49	96.70	97.62	–
NPV, %	100 ^{Aa}	54.79 ^{Bb}	100 ^{Aa}	86.96 ^{Ab}	–

Different uppercase letters represent significant differences between days ($P < 0.05$).

Different lowercase letters represent significant differences between methods ($P < 0.05$).

PPV: positive predictive value; NPV: negative predictive value.

4. Discussion

In this study, we demonstrated that using a commercial ELISA test to detect serum PAG concentrations was a highly accurate form of early pregnancy diagnosis in *Bos indicus* beef cattle. Our results are consistent with recent research examining the effectiveness of such tests in dairy taurine cows (Karen et al., 2015; Ricci et al., 2015). Specifically, PAG ELISA was 100% accurate in categorizing pregnant cows based on day 25 blood samples, compared with 93.9% (Romano and Larson, 2010), 94.6% (Sinedino et al., 2014), and 96.8% (Silva et al., 2007) sensitivity at 28 days post-TAI using the same test in taurine cows. Minor variations in these results are likely due to the fact that Zebu cows have higher baseline PAG concentrations than taurine cows (Mercadante et al., 2013). Because breed, fetus number, and fetus genotype can all influence PAG levels (de Sousa et al., 2003), these factors must be accounted during any cross-breed comparisons. Indeed, interspecific differences in gestational period could also lead to variation when PAG is detectable in the cow (Garbayo et al., 2008).

Several previous studies (Real et al., 2006; Romano et al., 2006) using fetal heartbeat detection with US also observed low sensitivity on day 25 post-TAI, similar to our findings. This lower diagnostic accuracy can be explained by difficulties in visualizing the gestational vesicle, along with ambiguities in interpreting image from early pregnancy. Specifically, the allantoic fluid can be confused with pathological or physiological fluids, and the gestational vesicle can only be clearly observed after 28 days of gestation (Barros and Visintin, 2001). In contrast, the high accuracy of PAG ELISA is related to the proteins' immunoreactivity across pregnancy. While PAGs are detectable from day 22, immunoreactivity increases considerably during days 24–28 and continues to rise until the last week of pregnancy. Thus, gestation diagnoses using PAG concentrations are the most accurate during this period (Green et al., 2005).

PAG ELISA and US specificity did not differ significantly. Our results corroborated previous findings using the same comparison method of $91.7 \pm 1.4\%$ specificity in dairy cattle between 25–45 days of gestation and $92.3 \pm 3.0\%$ specificity in beef cattle ($92.3 \pm 3.0\%$) between 29–56 days of gestation (Green et al., 2009). Further, diagnoses using qualitative commercial ELISA (97.1%) and quantitative immunometric ELISA (91.2%) yielded specificity similar to our observed values (Piechotta et al., 2011).

While ELISA PAG yielded some false positives, this may have attributed to some reasons. One of these could be the late embryonic mortality occurring around 25 days post mating. After pregnancy loss, PAG concentrations will inevitably decrease and disappear. However, because of the long half-life of this protein of approximately eight days (Mialon et al., 1993), the significant drop of PAG in the bloodstream may be delayed, compromising, even subtly, the accuracy of the test. The concentrations of PAG in cows with embryonic mortality are very similar to pregnant cows between 24 and 35 days, which makes it difficult to predict early onset of embryonic loss and to maintain the high accuracy of the early pregnancy tests (Szenci et al., 2000).

Another possibility is related to late luteolysis, since the presence of PAG is associated with the presence of the corpus luteum (Humblot, 2001). According to this author, because of luteal maintenance, about 20 to 30% of non-pregnant females have detectable PAG concentrations at 24 days post AI when compared to pregnant cows, increasing the number of false positives. Antiluteolytic signals and PAG levels are related at the beginning of gestation since both proteins are released by the trophoblast cells (Humblot et al., 1988a). Some factors can be speculated in relation to the accuracy of false positive results, as the use of inseminated cows once or twice before the experiment, in other words, a subfertile group of females (Humblot et al., 1988b).

During pregnancy, binucleate cells migrate from the trophoblast tissue to the uterine epithelium and the PAG produced by these cells exert distinct biological functions between the placental tissues (Touzard et al., 2013). This is an active process that requires a healthy

trophoblastic tissue, therefore, in cases of uterine infections or other mechanisms that support the maintenance of this tissue, the source of PAG is not maintained and the concentrations decrease allowing for misinterpretations during the diagnosis (Dobson et al., 1993).

PAG ELISA was highly accurate in detecting non-pregnant females, corroborating previous results of 100% negative predictive value in beef cattle (Green et al., 2009). However, our test was more accurate than a previous test in dairy cattle (94.7%; Romano and Larson, 2010) and two different ELISA tests (91.7% and 91.2%; Piechotta et al., 2011). That could be maybe due to the higher PAG levels in the Zebu females (Mercadante et al., 2013).

In contrast, US negative predictive value on day 25 (54.79%) was lower than previous findings of 67.5% on days 26–27 post-insemination (Szenci et al., 1998). Difficulties in observing the gestational vesicle on day 25 likely contributed to the discrepancy between studies. Supporting this explanation, the negative predictive value for US increased significantly on day 28 (86.4%), when the criterion for positive diagnosis was the presence of allantoic fluid. Nonetheless, we still obtained an inferior outcome compared with a previous US examination on day 28 post-TAI, which was 100% accurate in identifying non-pregnant cattle (Gradela et al., 2009). We believe the fluid amount inside the uterine lumen increased diagnosis difficulty and explains the high percentage of false negatives in our US tests, especially on day 25. Alternatively, embryos in some cows might be positioned behind endometrial folds (DesCôteaux et al., 2009), making them impossible to detect using US.

5. Conclusions

We demonstrated that the PAG ELISA technique is highly sensitive, specific, and accurate in early pregnancy diagnoses of *Bos indicus* beef cattle. Furthermore, PAG ELISA has a major advantage over methods such as US and transrectal palpation, considering the efficiency of the techniques, the time interval service – pregnancy diagnosis, and absence of manipulation of the uterus. Thus, we suggest that this method can be used safely to ascertain gestation status in Zebu cows, beginning from day 25 after mating.

Conflict of interest

The authors declare that they have no conflict interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2018.06.018.

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