



## 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, we 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 <sup>Aa</sup>	62.50 <sup>Bb</sup>	100 <sup>Aa</sup>	93.18 <sup>Ab</sup>	–
Specificity, %	92.86	95.24	92.86	95.24	–
PPV, %	96.70	96.49	96.70	97.62	–
NPV, %	100 <sup>Aa</sup>	54.79 <sup>Bb</sup>	100 <sup>Aa</sup>	86.96 <sup>Ab</sup>	–

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.

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