Original article

Accuracy of bovine pregnancy-associated glycoproteins (bPAGs) in the diagnosis of pregnancy: A comparative study of three pregnancy diagnostic methods

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Abstract

One of the effective reproductive management programs in dairy cattle is the accurate detection of pregnancy. A total of 204 non-descriptive cows were examined for pregnancy before slaughter in Sulaimani abattoir. Examinations were done by rectal palpation and enzyme-linked immunosorbent assay (ELISA) to measure the levels of progesterone and bovine pregnancy--associated glycoproteins (bPAGs) in their blood. Detection of a live conceptus in the uterus of slaughtered cows was used as the gold standard to determine the accuracy of the three pregnancy detection methods. The results showed that the accuracies of rectal palpation, progesterone assay, and bPAGs assay in the diagnosis of pregnancy were 87.2%, 84.8%, and 97.05%, respectively. The bPAGs assay scored the highest sensitivity (100%) for detection of pregnancy, followed by the progesterone assay (92.3%) and rectal palpation (84.6%). In addition, the specificity of the bPAGs assay was the highest (96.0%), while progesterone assay exhibited the lowest specificity (80.1%) and rectal palpation showed a specificity rate of (88.8%). In conclusion, the best method for the detection of either for early or late pregnancy in cows was the bPAGs assay, which gave the lowest number of false-positive and false-negative results.

Keywords: bPAGs, detection of pregnancy, progesterone, rectal palpation

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Introduction

Early and accurate diagnoses of pregnancy are essential factors for the implementation of proper management programs in livestock farming (Inchaisri et al. 2010). Fertility in a herd of dairy cattle is usually measured by the average calving interval, which is mostly influenced by the length of the time between calving and the next conception. A good way to increase the fertility rate in a herd is to reduce this interval, which in turn can be reduced by the early detection of the pregnant cows to separate those that have not conceived after service (Pieterse et al. 1990). Accurate identification of open cows following insemination is a key component in effective reproductive management to facilitate earlier re-insemination of the animals (Whitlock and Maxwell 2008). Thus, new methods to identify dairy cows that are nonpregnant after insemination will play a big role in management strategies to improve reproductive efficiency and profitability of dairy farms. Veterinary practitioners use different methods to detect open cows following insemination. These are classified into the managemental, clinical, laboratory, and ultrasonographical methods (Taverne and Noakes 2009).

Pregnancy-associated glycoproteins (PAGs) are secreted by the mononucleated and binucleated cells of the embryonic trophoblast and have been used to determine the status of pregnancy in cattle (Green et al. 2005). These proteins belong to the aspartic proteinases and constitute a large multigene family with approximately 100 genes that encode PAGs in cattle (Xie et al. 1997). Binucleate trophoblast cells are responsible for the expression of the majority of PAGs, even though all trophoblast cells are thought to express these glycoproteins (Green et al. 2000). Another method for the detection of pregnancy is based on serum progesterone concentration. This hormone is produced by corpus luteum and placenta and its level remains elevated during the gestation period. Pregnancy can be detected after 19-22 days of insemination using the progesterone assay (Skemesh et al. 1973). Transrectal palpation of the uterus is the most commonly implemented method for pregnancy detection after 30 days of insemination. The accuracy of this method depends on the experience and skills of the examiner and the size and age of the cows (Fricke et al. 2016). This study compared the accuracy of bovine pregnancy-associated glycoproteins (bPAGs), progesterone assay, and transrectal palpation in the detection of pregnant and nonpregnant cows. The accuracy of the three methods was compared based on the visual examination of the uterus of slaughtered cows at the abattoir.

Materials and Methods

Animals of the study

The study was carried out between September 2014 and April 2015. Cows that were brought to Sulaimani slaughterhouse, located in the city of Sulaymaniyah, Kurdistan Region, Northern Iraq, were used in the study. The age of the cows was determined via dental examination before being included in the study and animals younger than two years were excluded.

Rectal palpation and blood sampling

Palpation per rectum of the cows' reproductive systems was conducted by trained personnel. The palpation was performed prior to the slaughtering of the cows to examine the reproductive organs and determine the status of pregnancy. Meanwhile, blood samples were collected from each cow from the coccygeal vein and were put into separate tubes; one tube was a plain tube and the other was coated with ethylenediaminetetraacetic acid (EDTA). The blood samples were stored in a cooled box during their transport to the Clinical Pathology Laboratory of the Veterinary Teaching Hospital, College of Veterinary Medicine, University of Sulaimani. The transportation of the blood samples usually took less than two hours. After arrival into the laboratory, serum and plasma were separated by centrifugation at $1500 \times G$ for five minutes and were transferred into 2.5 mL-capacity Eppendorf tubes. The samples were then stored at -20°C until they were used.

Macroscopic examination of the genital system

Following rectal examination of cows older than two years, they were slaughtered in the slaughterhouse and the genital systems were immediately collected and examined for pregnancy. Detection of a conceptus in the uterine horn, after the uterus was opened, was used as the gold standard for the determination of pregnancy. The accuracy, specificity, and sensitivity of each of the other three methods were based on the results of the macroscopic visualization of a conceptus.

The fetuses were subjected to crown-rump length (CRL) measurement and the age of the fetus in days was calculated as $2.5 \times [21 + CRL]$ (Taverne and Noakes 2009). Rectal palpation is recommended for the detection of pregnancy from 30 days onward. Hence, cows which were pregnant for less than 30 days were not included in the study.

Table 1. Results of pregnancy detection methods.

Pregnancy detection method	Correct positive	Correct negative	False-positive	False-negative	Total
Rectal palpation	66 (32.4%)	112 (54.9%)	14 (6.9%)	12 (5.9%)	204 (100%)
bPAGs assay	78 (38.2%)	121 (59.3%)	5 (2.5%)	0	204 (100%)
Progesterone assay	72 (35.3%)	101 (49.1%)	25 (12.3%)	6 (2.9%)	204 (100%)
Macroscopic examination	78 (38.2%)	126 (61.8%)	0	0	204 (100%)

There were significant differences ($p<0.001$) between the values of bPAGs and the other two methods. No significant differences were present between the values of rectal palpation and progesterone assay.
Test: Chi-square.

Progesterone concentration assay

Progesterone concentration in the serum was determined using a progesterone ELISA kit (Monobind Inc., USA) and the absorbance was read using an ELx800™ Absorbance Microplate Reader (BioTek® Instruments, Inc., USA). The instructions of the kit manufacturer were followed in the procedure.

Bovine pregnancy-associated glycoproteins detection (bPAGs assay)

The concentration of bPAGs in the plasma of the test animals was determined by capture ELISA using a commercial kit from IDEXX Inc., USA. The procedure used was according to the kit manufacturer's instructions.

Statistical analysis

The results of pregnancy detection by the rectal examination, progesterone assay, and the concentration of bPAGs in the plasma were divided into four types: correct positive, false positive, correct negative, and false negative. The sensitivity of each of the three methods was calculated as $100 \times$ [correct positive $\div$ (correct positive + false negative)]. The specificity of the tests was calculated as $100 \times$ [correct negative $\div$ (correct negative + false positive)]. Furthermore, positive and negative predictive values were calculated using the following formulas:

Positive predictive value $= 100 \times$ [correct positive $\div$ (correct positive + false positive)] /1/

Negative predictive value $= 100 \times$ [correct negative $\div$ (correct negative + false negative)] /2/

The accuracy of each of the methods for pregnancy detection was determined as $100 \times$ [(correct positive + correct negative) $\div$ (correct negative + correct positive + false positive + false negative)] (Martin et al. 1987).

Data were tested for normality using the Shapiro-Wilk test. The cut-off value of plasma progesterone for discrimination between pregnant and non-pregnant cows was determined using Receiver-Operating Curve (ROC).

Chi-square was used to compare the differences in the number of correct positive, false positive, correct negative, and false-negative results among the three methods.

All the statistical analyses of data were performed using version 22.0 of the Statistical Package for Social Sciences (SPSS) software (by IBM, USA). Differences were considered statistically significant when the probabilities were <0.05.

Results

Animals of the study

The study was conducted on the cows that were brought for slaughtering in the Sulaimani slaughterhouse. The slaughterhouse was visited twice a week for about seven months, from September 2014 to April 2015. During that period, a total of 204 non-descriptive cows that were older than two years were brought to the slaughterhouse and were examined for pregnancy. Detection of pregnancy was accomplished through rectal examination, macroscopic (visual) examination of the reproductive system, progesterone assay, and bPAGs assay.

Macroscopic examination of the genital system

Visual examination of the genital system, after the cows were slaughtered, served as the gold standard to correctly determine whether the animal was pregnant or not. Seventy-eight of the slaughtered cows were pregnant (Table 1) and the remaining 126 cows were non-pregnant. The ages of the conceptuses in the pregnant animals were normally distributed throughout the months of the gestation (Table 3).

Detection of pregnancy by rectal palpation

Sixty-six cows of 204 (32.4%) were diagnosed as being pregnant through rectal palpation. However, the correct number of pregnant cows was 78/204 (38.2%) after confirmation by visual observation

Table 2. Performance values of the different methods of pregnancy detection.

Pregnancy detection method	Performance values (%)				
	Sensitivity	Specificity	PPV	NPV	Accuracy
Rectal palpation	84.6	88.8	94.2	90.3	87.2
bPAGs assay	100	96.0	93.9	100	97.5
Progesterone assay	92.3	80.1	74.2	94.3	84.8

PPV = positive predictive value
NPV = negative predictive value

Table 3. Distribution of pregnancy throughout the gestation period.

Pregnancy detection method	Gestation time (month)															
	2nd		3rd		4th		5th		6th		7th		8th		9th	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Rectal palpation	3	5	5	5	12	1	11	1	11	0	9	0	5	0	10	0
bPAGs assay	8	0	10	0	13	0	12	0	11	0	9	0	5	0	10	0
Progesterone assay	8	0	10	0	13	0	12	0	11	0	9	0	5	0	4	6
Macroscopic examination	8	0	10	0	13	0	12	0	11	0	9	0	5	0	10	0

+: cows correctly diagnosed as being pregnant (correct positive)
-: cows incorrectly diagnosed as being non-pregnant (false negative)

of a live conceptus in the animals' uteruses (Table 1). The rectal palpation method of pregnancy detection gave the highest rate of false-negative results (5.9%), compared to bPAGs (0.0%) and progesterone (2.9%) assays. The accuracy of rectal palpation in the detection of pregnancy was 84.7%, while the sensitivity was 78.5% and the positive predictive value was 87.2% (Table 2). Rectal palpation was the least sensitive in the detection of pregnancy, especially in the second month of gestation (Table 3).

Detection of pregnancy by bovine pregnancy-associated glycoproteins assay

The bPAGs assay was the most accurate, sensitive, and specific among the three methods of pregnancy detection (Table 2). The test resulted in the lowest false positive and false negative results, compared to rectal palpation and progesterone assay. Only five cases of false-positive were recorded. Three of the cases had pyometra and two cases of false-positive were from cows that had recently given birth. Pregnancy detection using bPAGs assay resulted in the highest rate of correct diagnoses. It was shown that all pregnant cows (78) were correctly diagnosed as being pregnant.

Detection of pregnancy by progesterone assay

The ROC analysis indicated that a serum progesterone level of 5.1 ng/mL was the cut-off value to diagnose a pregnant cow (Figure 1). The results of the progesterone assay were divided into four types: correct positive, correct negative, false positive and false negative (Table 1). Seventy-two cows were correctly diagnosed as being pregnant, while only 6 cows were false negatives. The false-negative results were scored in the ninth month of pregnancy (Table 3). All the 68 pregnant cows that were in their 2nd to 8th month of gestation were correctly diagnosed using the progesterone assay. This indicates that the progesterone assay was 100% sensitive in the diagnosis of pregnancy from the second to the eighth month of gestation. The progesterone assay was 92.3% sensitive and 80.1% specific in the diagnosis of pregnancy throughout the gestation period of the tested cows.

Discussion

This study compared the accuracy of rectal palpation, progesterone assay, and bPAGs assay in the detection of pregnancy in non-descriptive cows. The accuracies of the three methods were compared based on the results obtained from the macroscopic examination of the genitalia of the slaughtered cows. Detection of a viable fetus in the uterus of a slaughtered cow is considered the gold standard for the determination of pregnancy, to which, other methods of pregnancy can be compared (Giraldo et al. 2010).

Palpation of the genital system through the rectum is the most common method for the detection of pregnancy, especially after the second month of gestation. However, rectal palpation is not an accurate method of pregnancy detection in the first month of the gesta-
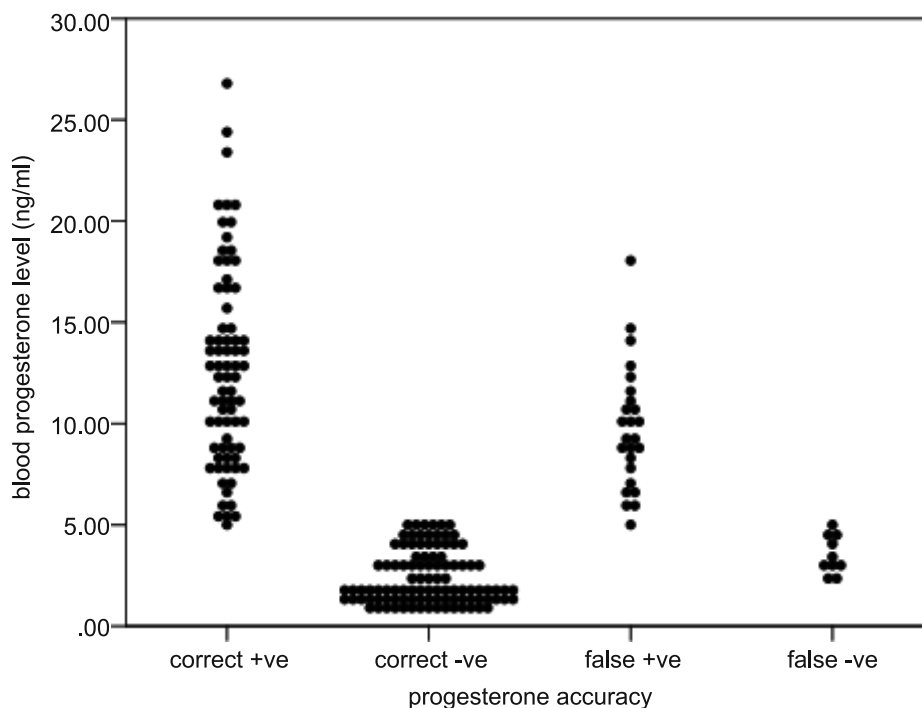
Fig. 1. Blood progesterone concentration of 5.1 ng/mL was considered as the cut-off value to determine pregnant and non-pregnant cows.

tion period. Moreover, abortion and fetal abnormalities can occur when rectal palpation is performed before 30 days of gestation (Romano et al. 2016). For these reasons, the accuracies of the three methods in our study were compared starting from the second month of gestation.

The accuracy of rectal palpation in the detection of pregnancy throughout the gestation period was 87.2%. The lower accuracy rate of this method in the second and third months of gestation has led to a decreased overall accuracy and specificity of the method when it was compared with the bPAGs assay. Other studies, however, reported accuracy levels of rectal palpation as high as 92.5% (Reimers et al. 1985) and 99.0% (Badtram et al. 1991). Factors such as the examiner's experience, the parity, age, and size of the dam, and the volume of fluid inside the fetal sacs can influence the degree of sensitivity of the method (Taverne and Noakes 2009).

The level of progesterone in the serum was also used as a method for the detection of pregnancy. The threshold level of the serum progesterone concentration between pregnant and nonpregnant cows was determined by Skemesh et al. to be 2.1 ng/mL (Skemesh et al. 1973). However, the threshold in the current study was estimated to be 5.1 ng/mL. Factors such as the animals' age, genetic merit, and the weather probably influence the serum progesterone level. The level of progesterone is higher in heifers and young lactating cows than in older animals (Bech-Sàbat et al. 2008). Samples taken in cold seasons contain a higher level of the hormone (Satheshkumar et al. 2015). It was reported that genetic factors probably influence the size of the corpus luteum and the level of progesterone (Moore et al. 2014). The progesterone assay resulted in the highest incorrect identification of nonpregnant cows. The reason is that progesterone is produced by the corpus luteum and placenta and, therefore, it is not restricted to pregnancy (Senger 2012). The accuracy of the test was 84.8%, which was close to the results of Samsonova et al. who reported an accuracy level of 87.0% (Samsonova et al. 2014). The assay resulted in 72/78 correct identification of pregnant cows. The six false-negative results reported by the test were in the last month of gestation.

Pregnancy-associated glycoproteins are synthesized in the fetal cotyledons and released into the maternal blood (Zoli et al. 1992). These glycoproteins can be used for pregnancy diagnosis as early as 21 days after artificial insemination in some animals and after 30 days in all pregnant cows (Perényi et al. 2002, Reese et al. 2018). The level of bPAGs progressively increases to reach the peak plasma level about one week before parturition (Zoli et al. 1992).

In the current study, five of the tested samples gave false positive results, of which, two samples were from cows that had recently given birth. The level of bPAGs peaks at the time of calving and gradually decreases to its lowest concentration at 80 days postpartum, which could probably give false-positive results (Kiracofe et al. 1993). The high serum levels of the glycoprotein in the two cases were probably from the previous calv-

ing. Three false-positive results were from cows with pyometra. These animals could have had a recent abortion, which resulted in a high serum level of bPAGs. The estimated half-life of the bPAGs is about seven days, which means the level of the glycoproteins may remain high enough to be detected a few weeks after abortion (Semambo et al. 1992). The bPAGs assay resulted in the highest number of correct positive (78/78) and correct negative (121/126) results and it was the most accurate method for pregnancy detection in the first month of gestation. This glycoprotein gives the most accurate results for the detection of pregnancy.

Conclusions

The accuracy of the bPAGs assay in the detection of pregnancy was investigated. Slaughtered pregnant cows at the Sulaimani abattoir were used to macroscopically detect the existence of a live conceptus inside the uterus of these animals. Observation of a live fetus provides the best diagnostic confirmation method of pregnancy and the method was used as the gold standard to compare the other pregnancy detection methods in this study. The bPAGs assay provided the highest levels of accuracy, sensitivity, and specificity in the detection of pregnancy. The bPAGs assay was superior to the progesterone assay and palpation per rectum starting from as early as 30 days of gestation. We conclude that the bPAGs assay is the best technique in the detection of pregnancy, especially after the first month of the gestation period.

Authors contribution

All authors contributed equally to this study, including experimental design, collection and interpretation of data, statistical analysis, and manuscript preparation.

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