Early detection of multifetal pregnancies in Alpine Goats using pregnancy-associated glycoprotein (PAG) concentrations in milk

Y.T. Chen[1], M.K. Yang[1], Y.H. Yeh[1], Y.H. Chen[1], J.W. Shiau[1], J.F. Huang[2] and P.A. Tu[1]

[1]Northern Region Branch, Livestock Research Institute, Ministry of Agriculture, No.207-5 Pitoumian, Xihu Township, Miaoli 368003, Taiwan
[2]Taiwan Livestock Research Institute, No.112, Farm Road, Hsinhua,Tainan 71246, Taiwan
Corresponding Author: tpa@mail.tlri.gov.tw

Abstract

The nutritional metabolic demands and foetal development needs of pregnant goats carrying multiples (≥3 foetuses) are significantly different from those of single and twin pregnancies, including requirements for energy, protein, and minerals. Early prediction of the number of foetuses can allow for timely adjustment of management practices for goats pregnant with multiples. In this study, milk samples from 348 pregnant Alpine goats were collected and analysed for PAG concentrations during early (10–43 days), mid (49–78 days), and late (85–94 days) pregnancy. To evaluate the number of foetuses in Alpine goats during early pregnancy and to assist dairy farmers in corresponding reproductive management, receiver operating characteristic (ROC) curve analysis was used to determine the sensitivity, specificity, and area under the curve (AUC) for different PAG concentration thresholds at various days of pregnancy. In this research, the fatal number of 1, 2, and 3 or more kids accounted for 21%, 71%, and 8% of total pregnant goats, respectively. The results showed that at 49 days of pregnancy, a milk PAG threshold of 1.208 for determining multiple pregnancies had a sensitivity of 100%, a specificity of 80.6%, and an AUC of 0.903; at 57 days of pregnancy, a milk PAG threshold of 2.643 resulted in a sensitivity and specificity of 100% with an AUC of 1. Moreover, the Youden index at 57 days was higher than at 49 days (1 vs. 0.806). We then used Canonical Discriminant Analysis (CDA) to evaluate the significant differences between the groups of multiple and single or twin pregnancies based on different milk PAG concentration thresholds and days of pregnancy. Starting from 49 days of pregnancy, the Wilk's Lambda value was 0.786, indicating a significant difference in milk PAG concentrations between goats with multiples and those with single or twin foetuses (P<0.01); at 57 days of pregnancy, the Wilk's Lambda value was 0.104, denoting a highly significant difference (P<0.001). It is concluded that 49 to 57 days of pregnancy are effective timepoints to use milk PAG concentrations to differentiate the number of foetuses in pregnant Alpine goats.

Keywords: Alpine goats, pregnancy-associated glycoprotein, foetal number. Presented at the ICAR Anual Conference 2024 in Bled at the Session 7: Breeding for agroecological transition in sheep and goats.

Introduction

Nutritional metabolism and foetal development requirements of goats carrying multiple foetuses (≥ 3 foetuses) are significantly different from those of single and twin pregnancies, including energy, protein, and mineral requirements, and the energy demand might gradually increase with gestational days (El-Sayed *et al*., 2022; Mongini and Van Saun, 2023). Insufficient energy intake may cause increased incidence of ketosis and toxaemia in ewes (Ji *et al*., 2023), so early determination of pregnancy numbers in ewes and providing appropriate care might reduce the incidence of disease (Dinç *et al*., 1994).

Pregnancy-associated glycoproteins (PAG) are members of the inactive aspartic proteinase family and secreted by binucleated cells in the ruminants placental nutritive layer (Singh *et al*., 2019a). Plasma caprine pregnancy-associated glycoprotein (caPAG) concentration started to increase at 22 days of gestation, reached the peak at 45 days of gestation, and rapidly declined after parturition, reaching baseline at 14 days postpartum (Singh *et al*., 2019a). Additionally, plasma PAG concentration in ewes carrying twins was significantly higher than those carrying singles at 28 days of gestation (Singh *et al*., 2019a), with concentrations in twins being 1.4-1.8 times higher than those in singles in the mid and late gestation (Singh *et al*., 2019a). As above, it is evidence that plasma PAG concentration is influenced by gestation and number of foetuses.

The objective of the experiment is to collect daily milk samples from Alpine goats after artificial insemination to measure PAG concentration in milk, determine the threshold for differentiating the number of foetuses by milk PAG concentration and confirm the optimal time point as a basis for foetus's number prediction.

Material and methods

Management of experimental animals and collection of milk sample

The data in this study were derived from 348 Alpine multiparous goats (3-5 pregnancies). Pregnancy detection was performed by ultrasound (ALOKA Prosound 2, Japan) between days 45 and 50 post-breeding. Pregnancy was confirmed based on the visualization of embryos, foetal membranes, and foetuses via ultrasound. Goats confirmed pregnant would advance to the next experiment, and the number of foetuses was recorded. The proportion of singles, twins and triplets are 21%, 71%, and 8%, respectively. Milk samples were collected from 10 days post-breeding and weekly until day 94 of pregnancy. Collected 1 mL of milk into sterile centrifuge tubes before milking and stored at 4°C before being sent to the milk testing laboratory of the Northern Branch of the Livestock Research Institute, Ministry of Agriculture. Samples were kept at room temperature before testing.

Analysis of PAG concentration in milk samples

The measurement of PAG concentration in milk samples was performed by commercially available antigen capture enzyme-linked immunosorbent assays (ELISA) (IDEXX Laboratories, Inc., Westbrook, Maine, USA). There are 2 wells for positive control (PC) and 2 wells for negative control (NC) in each 96-well plate. Incubated the 96-well plate in a 37°C oven with agitation for 2 hours, and measured the optical density (OD) of samples in 96-well plate at 450 nm and 630 nm wavelengths by a SpectraMax® Absorbance Reader CMax Plus (USA), and calculated the average values of PC and NC as PC mean and NC mean. The validity of the results was confirmed by the difference between PC mean and NC mean being more than 0.5 and NC mean being below 0.2. The PAG concentration in milk was calculated by subtraction of the NC mean with the OD value of the samples as PAG (sample-negative) value. A difference more than 0.25 (including 0.25) was considered as positive, indicating pregnancy.

Statistical analyses

For statistical analysis of milk PAG concentration in Alpine goats at different pregnancy days, receiver operating characteristic (ROC) analysis were used to establish a predictive model for multiple pregnancies. The area under the curve (AUC) was used to determine the optimal prediction time point for the highest accuracy in predicting multiple pregnancies in Alpine goats. The optimal cutoff point was determined based on the maximum value of the Youden index = sensitivity + specificity - 1. Subsequently, canonical discriminant analysis (CDA) coefficients were evaluated based on linear combinations of various variables to confirm the accuracy between different sampling time points (10, 33, 43, 49, 57, 65, 71, 78, 85, and 94 days of pregnancy). Wilk's Lambda value was to assess overall significance of multiple pregnancies on PAG concentration in Alpine goat milk at different pregnancy days. Wilk's Lambda value was ranging from 0 to 1. When Wilk's Lambda value is closer to 0, it can be considered that multifetal pregnancies have a significant influence on Alpine goat milk PAG concentration at different pregnancy days.

Results

Evaluation of the ROC curve on milk PAG concentration thresholds at different pregnancy days in Alpine goats

To confirm the number of pregnancies in Alpine goats in early gestation, this experiment collected milk samples from 348 pregnant Alpine goats during early pregnancy (10-43 days), mid-pregnancy (49-78 days), and late pregnancy (85-94 days). This experiment calculated the milk PAG concentration thresholds at different pregnancy days by ROC curve to verify the accuracy of multifetal pregnancies determination. Results showed that while the milk PAG concentration threshold is 1.208, the sensitivity is 100%, specificity is 80.6% and an AUC is 0.903 at 49 days of pregnancy. At 57 days of pregnancy, the milk PAG threshold is increase to 2.643, the sensitivity and specificity are both 1, and AUC is 1. Furthermore, using the Youden index as the criterion for determining the optimal cutoff timepoints. Results showed that Youden index values are of 0.29, 0.67, 0.806, and 0.806 at 10, 33, 43, and 49 days of pregnancy, respectively. The Youden index values were all 1 at 57, 65, 71, 78, 85, and 94 days of pregnancy. This indicated that the most effective timepoints to confirm multifetal pregnancies by milk PAG is between 49 and 57 days of pregnancy.

Table 1. Result of receiver operating characteristic curve (ROC curve) analysis [sensitivity (Se), specificity (Sp) and area under curve (AUC)] of milk pregnancy-associated glycoprotein (PAG) ELISA assessment for determination of triple foetuses based on different threshold values of circulating PAG concentration (S-N) at different days of gestation in Alpine goats.

Days of pregnancy	PAG threshold values	Se (%)	Sp (%)	AUC and P value	95% Confidence interval	
					Lower bound	Upper bound
10	0.024	100.0	29.0	0.452 (P = 0.779)	0.113	0.790
33	0.300	100.0	67.7	0.720 (P = 0.006)	0.563	0.878
43	0.446	100.0	80.6	0.828 (P < 0.001)	0.696	0.960
49	1.208	100.0	80.6	0.903 (P < 0.001)	0.785	1.000
57	2.643	100.0	100.0	1.000 (P < 0.001)	1.000	1.000
65	3.326	100.0	100.0	1.000 (P < 0.001)	1.000	1.000
71	3.421	100.0	100.0	1.000 (P < 0.001)	1.000	1.000
78	3.756	100.0	100.0	1.000 (P < 0.001)	1.000	1.000
85	2.126	100.0	100.0	1.000 (P < 0.001)	1.000	1.000
94	3.146	100.0	100.0	1.000 (P < 0.001)	1.000	1.000

Evaluation of CDA on milk PAG thresholds at different pregnancy days in Alpine goats to differentiate the number of foetuses

To assess the milk PAG concentration of multiple pregnancies (twins or triplets) versus single or twin pregnancies in Alpine goats at different gestational days, CDA was conducted. Results showed that on 49 days of pregnancy, the Wilk's Lambda value was 0.786, indicating a significant difference in milk PAG concentration between multiple pregnancies and single or twin pregnancies ($P < 0.01$). On 57 days of pregnancy, the Wilk's Lambda value was 0.104, indicating an extremely significant difference in milk PAG concentration between multiple pregnancies and single or twin pregnancies ($P < 0.001$). It demonstrated that from 49 to 57 days of pregnancy, milk PAG concentration can effectively differentiate between multiple pregnancies and single or twin pregnancies.

Table 2. Canonical discriminant analysis (CDA) results for discrimination of single or twin foetuses from triple foetuses with milk pregnancy-associated glycoprotein (PAG) enzyme-linked immunosorbent assay (ELISA) assessment at different days of pregnancy in Alpine goats

Canonical discriminant function coefficients		Days of pregnancy									
		10	33	43	49	57	65	71	78	85	94
Wilk's Lamba value		0.996	0.993	0.996	0.786**	0.104***	0.179***	0.255***	0.317***	0.278***	0.068***
Unstandardized coefficients		5.648	4.110	2.276	2.369	4.213	2.689	2.198	1.826	3.469	4.802
Group centroids	Single or dual foetuses	0.020	-0.025	-0.020	-0.157	-0.885	-0.647	-0.515	-0.443	-0.486	-1.115
	Triple foetuses	-0.207	0.253	0.208	1.627	9.150	6.684	5.324	4.581	5.022	11.526
Eigenvalue		0.004	0.007	0.004	0.272	8.608	4.594	2.915	2.158	2.593	13.659
% of variance		100	100	100	100	100	100	100	100	100	100
Canonical correlations		0.066	0.081	0.067	0.463	0.947	0.906	0.863	0.827	0.850	0.965

[1] ** indicates a significant difference (P < 0.01).
[2] *** indicates a highly significant difference (P < 0.001).

Discussion

Goats carrying multiples requires precision feeding management to face the metabolic demands. If goats carrying multiples didn't intake sufficient energy to meet metabolic demands, lipid mobilization is an adaption, resulting in the increase of blood non-esterified fatty acids (NEFA) and -hydroxybutyrate (BHBA) concentration (2.1 and 3.7 times, respectively) than those carrying single pregnancies before delivery (Moallem *et al*., 2012). Excessive related metabolites could be transferred into ketones in the liver, increasing the incidence of pregnancy toxaemia in ewes (Ji *et al*., 2023). PAG has been studied as an indicator for early pregnancy diagnosis and even predicting the number of foetuses in ruminants (Hussein *et al*., 2017). Therefore, detecting the changes in milk PAG concentration during pregnancy might be a way to differentiate the number of foetuses, improve the nutritional management of ewes carrying multiples and reduce the incidence of disease.

In the experiments showed that at 10, 33, and 43 days of pregnancy, the sensitivity was 100%, but the specificity was only 29.0%, 67.7%, and 80.6%, respectively, with AUC of 0.452, 0.720, and 0.828, indicating that the accuracy of detecting the number of foetuses at 10, 33, and 43 days of pregnancy was low. At 49 days of pregnancy, with a milk PAG concentration threshold of 1.208, the sensitivity was 100%, specificity was 80.65%, and AUC was 0.903; while at 57 days of pregnancy, the milk PAG concentration threshold was 2.643, with both sensitivity and specificity at 100% and AUC at 1, suggesting that at 57 days of pregnancy, goats carrying multiples could be identified accurately. Yang *et al*. (2022) showed that milk PAG concentration in goats carrying multiples started increasing at 49 days of pregnancy compared to single or twin pregnancies. In the present study, the determination of milk PAG concentration at 49 days of pregnancy has showed a certain degree of accuracy (80.65%), in agreement with the results in Yang *et al*. (2022). Although the threshold in the present study was higher than Singh

et al. (2019a)'s value of 0.830, it might attribute to the threshold in Singh *et al*. (2009a) based on PAG in blood and the differentiation between single and twin pregnancies. Furthermore, the target breed in Singh *et al*. (2019a) was Indian Barbari goats, whereas the present study was focused on Al-Bayda goats, contributing to differences in PAG concentrations (De Carolis *et al*., 2020). Additionally, we measured milk PAG concentration, with the threshold of 1.208 to differentiate between singles and twins or multiples. Some studies showed that PAG in blood was about 1.9-2.0 times higher than in milk (Singh *et al*., 2019b), indicating that blood PAG threshold of 0.830 divided by 2 was the milk threshold of about 0.415. The value (0.415) was close to the value of singles and twins in Yang *et al*. (2022), presuming that the number of foetuses might be a main factor of PAG concentration threshold. Moreover, it was noteworthy that the timepoints which Singh *et al*. (2019a) distinguished the number of foetuses was over 45 days of pregnancy. This result was consistent with Yang *et al*. (2022), pointing out PAG concentration in mid pregnancy was related to number of foetuses. Therefore, it could serve as evidence to determine the timepoints of the number of foetuses (49 to 57 days of pregnancy).

In the present study, Canonical Discriminant Analysis (CDA) was used to measure milk PAG concentration at different pregnancy days to distinguish between singles, twins, and multiples. Results showed that at 49 days of pregnancy, Wilk's Lambda value was 0.786, indicating a significant difference ($P < 0.01$) in milk PAG concentration between goats with single or twin pregnancies and with multiple pregnancies. At 57 days of pregnancy, Wilk's Lambda value decreased to 0.104, indicating an extremely significant difference ($P < 0.001$) in milk PAG concentration between goats with singles or twins and with multiples. This suggested that milk PAG concentration could start to differentiate the number of foetuses between 49 and 57 days of pregnancy. The analysis by Youden index to determine the optimal detection timepoints was also between 49 and 57 days of pregnancy. Also, other studies indicated that at 45 days of pregnancy, it was a significant difference in serum PAG concentration in singles, twins and triplets, with concentrations of 58.23 ± 2.64, 48.55 ± 2.86, and 34.77 ± 1.53 ng/ml, respectively.

These results suggested that serum PAG concentration can be used to differentiate the number of foetuses at 45 days of pregnancy (Hussein *et al*., 2017). Similarly, the difference of PAG concentration between the number of foetuses, especially in multiples, was also mentioned in Yang *et al*. (2022). However, Hussein *et al*. (2017) measured goats serum PAG concentration, while Yang *et al*. (2022) used milk samples from pregnant goats. Although the target substances in these two studies were different, there was a strong positive correlation ($R^2 = 0.64$) between blood and milk PAG concentrations (Singh *et al*., 2019a). Additionally, both blood and milk PAG concentrations showed high accuracy in determining pregnancy after 32 days of pregnancy, with accuracies 92% and 89%, respectively. Based on the studies, the timepoints for blood and milk PAG concentrations to determine the number of foetuses were quite close, indicating that milk PAG concentration can be used to determine the number of foetuses between 49 and 57 days of pregnancy.

Conclusion

According to the above, at 49 days of pregnancy, the milk PAG concentration could be used to detect for multiple pregnancies in goats. Additionally, at 57 days of pregnancy, milk PAG concentration could differentiate between single and multiple pregnancies in goats. Therefore, 49 to 57 days of pregnancy is the most ideal time for detecting milk PAG concentration and determining the number of foetuses in Alpine goats. These findings will enable farmers to confirm the number of foetuses in early pregnancy, apply for precise feeding and management and reduce the incidence of metabolic disorders in goats.

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