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Investigation of pregnancy-associated glycoproteins (PAGs) by means of an enzymoimmunoassay (ELISA) sandwich kit for pregnancy monitoring in sheep

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Abstract. The aim of the present study was to investigate the use of a PAGs ELISA-Sandwich kit (Ref. code E.G.7. CER. Marloie, Belgium) for both early pregnancy diagnosis (in Sardi sheep) and the pregnancy follow up (in Boujaâd and Boujaâd x D'man sheep). In Sardi breed, plasma samples were obtained from pregnant ewes (n = 17) from day 18 to 30 of gestation at 2 days interval. In Boujaâd (n = 8) and Boujaâd x D'man (n = 20) the blood samples were weekly collected from the first week of gestation till the 4th week after lambing. The PAG concentrations were determined by a sandwich-ELISA based on purified bovine PAG (boPAG-67 kDa) as a standard, the antiserum raised against caprine PAG (caPAG-55+62 kDa) as a capture antibody (1/40 000) and antiserum raised against purified PAG from buffalo (AS 859) as detection antibody (1/32 000). The Avidin-HRP and TMB were used to reveal reactions. Ewes were assumed to be pregnant when PAG concentrations were higher than 0.8 ng/ml. Results showed that in Sardi sheep it is possible to detect all ewes as pregnant as early as on 24 days of gestation. In Boujaâd and Boujaâd x D'man the PAG concentrations reached the higher values just before lambing. This value is higher in ewes carrying more than one lamb than in those carrying a single lamb. To conclude, the present study shows that the ELISA kit used for early pregnancy diagnosis in sheep as well as for pregnancy fellow up could be a good alternative to the radioimmunoassay RIA in countries where this last technique is hard to set up.

Keywords. ELISA – Sandwich – PAGs – Moroccan sheep – Pregnancy diagnosis.

Investigation des protéines associées à la gestation chez la brebis par l'utilisation d'un kit ELISA-Sandwich

Résumé. *L'objectif du présent travail a été d'étudier l'utilisation d'un kit ELISA sandwich (Réf. EG7 Code. CER. Marloie, Belgique) pour le diagnostic précoce de la gestation (chez la brebis Sardi) et le suivi de la gestation (chez la brebis Boujaâd et Boujaâd x D'man). Chez la race Sardi, des échantillons de plasma ont été obtenus à partir de brebis gestantes (n = 17) du 18ième au 30ième jour de gestation à 2 jours d'intervalle. Chez les brebis Boujaâd (n = 8) et Boujaâd x D'man (n = 20) des échantillons de sang ont été collectés une fois par semaine à partir de la première semaine de gestation jusqu'à la 4ième semaine après agnelage. Les concentrations de PAG ont été déterminées par ELISA Sandwich basé sur une PAG bovine (boPAG-67 kDa) comme standard un antisérum dirigé contre la PAG caprine (caPAG55- 62 kDa) comme anticorps de capture (1/40 000) et un antisérum dirigé contre les PAGs de buffle (As 859) comme anticorps de détection (1/32 000). L'avidine-HRP et le TMB ont été utilisés pour la révélation des réactions. Les brebis ont été considérées gestantes lorsque les concentrations de PAG étaient supérieures à 0,8 ng / ml. Les résultats ont montré que chez la brebis Sardi, il est possible de détecter toutes les brebis gestantes dès le 24ième jour. Chez les brebis Boujaâd et le Boujaâd x D'man, les concentrations en PAG atteignent les valeurs les plus élevées juste avant l'agnelage. Ces valeurs sont plus élevées chez les brebis à portée double que chez celles à portée simple. En conclusion, la présente étude montre que le kit ELISA-PAG utilisé pour le diagnostic de gestation précoce ainsi que pour le suivi des gestations pourrait être une bonne alternative au dosage radioimmunologique dans des pays où l'utilisation de cette technique est difficile.*

Mots-clés. *ELISA-Sandwich – PAGs – Ovin Maroc – Diagnostic de gestation.*

I – Introduction

The pregnancy-associated glycoproteins (PAG), also named pregnancy-specific protein B (PSPB), were initially characterized as placental antigens detectable in the maternal circulation of pregnant cows (Butler *et al.*, 1982; Zoli *et al.,* 1992). From 1988, the identification of placental antigens immunologically related to bovine PAG/PSPB in the peripheral circulation of pregnant ewes (Ruder *et al.,* 1988) and the hope to develop an ovine homologous radioimmunoassay (RIA) have encouraged their isolation and characterization in this species. The measurement of PAG/PSPB molecules in peripheral circulation of ovine species can give useful information to develop appropriate feeding strategies for pregnant females and to insure the mother's requirements and the fetuses growth to avoid metabolic disorders associated to pregnancy (El Amiri *et al.,* 2003).

Blood and milk concentration of PAGs were initially measured by radioimmonoassy (El Amiri *et al.,* 2003; Vandaele *et al.,* 2005) and the results have been compared with those of ultrasonography (Karen *et al.,* 2003). More recently, an ELISA for PAG became available. It provides a quantitative pregnancy classification based on measurement of those molecules in the serum of pregnant ruminants. However, these assays have not been tested in Moroccan ewes and never have been compared to those obtained by RIA. Therefore, the aim of the present work was to study the efficiency of an ELISA sandwich kit for early pregnancy diagnosis and pregnancy follow up in Moroccan sheep.

II – Material and methods

1. Animals and blood sampling

Tow experiments were carried out for PAG detection. The first one concerned the early pregnancy diagnosis in Sardi sheep (n = 17 pregnant and 6 non pregnant) and the second focused on the pregnancy fellow up in Boujaâd (n = 8) and Boujaâd X D'man sheep (n = 12). The Sardi sheep belongs to a private farm located at Ouled Said region (Settat province). The Seventy ewes were synchronized by flurogestone acetate (20 mg, Chronogest; Intervet International, France) intravaginal sponge insertion for 14 days. At the time of sponge removal, ewes received 300 IU of eCG (Folligon; Intervet International France). All ewes were naturally mated. The day of first mating was considered as day 0. Blood samples (5 ml) were collected from each ewe at days 0, 18, 20, 22, 24, 26, 28 and 30 after mating. For Boujaâd and Boujaâd X D'man, the samples were collected from the first week of mating to the 4th week of post partum. They were withdrawn from the jugular vein into EDTA vacutainer tubes which were put into a cool box until centrifugation. The plasma was separated after collection by centrifugation at 1500 x g for 20 min, and then stored at -20°C until assayed for PAG concentrations.

2. PAG measurements by RIA system

Due to its high sensitivity and specificity, and because the RIA is more quantitative and more accurate to investigate subtle differences in PAG concentrations, the RIA was used in this study as a control. Briefly, all PAG concentrations were determined by means of a homologous radioimmunoassay (RIA) with the antisera (R780) raised against ovPAG$_{57+59}$ (1:400 000) and bovine PAG as standard and tracer. The purified boPAG was radiolabelled by chloramine T using ^{125}I. The assay was developed in Tris-BSA buffer. All measurements were performed in duplicate, in polystyrene tubes, under equilibrium conditions at room temperature. Samples with PAG concentrations beyond or above the range of the standard curve of the assay were re-assayed in a non-preincubated system or diluted.

3. PAG measurements by ELISA system

The ELISA technique was a sandwich ELISA and employed C96 Maxisorp NUNC – immuno plates coated with rabbit anti-PAG serum (As-707 raised against $caPAG_{66+62kDa}$ used at 1:40 000). The detecting antibody was rabbit anti-PAG IgG (As-859 raised against buffalo PAG used at 1:32 000) as biotin-conjugate. The standard curve was performed after diluting the $boPAG_{67kDa}$ at 0.4, 0.6, 0.8, 1.5 and 2 ng/ml. The enzyme substrate was 3,3',5,5' tetramethylbenzidine.

4. Statistical analysis

A mixed model was fitted to the PAG concentration data including ewe as random effect and time (week 1 to week 24), ewes's weight, litter size, sex of the foetus, total lambs's weight as categorical fixed effects (SAS version 8).

III – Results and discussion

1. Early pregnancy diagnosis

Results showed that in Sardi sheep 21.42%, 35.71% and 71.42% are detected as pregnant respectively on days 18, 20 and 22. it is possible to detect all ewes as pregnant as early as on day 24 of gestation (Table 1). The highest level of PAG is reached on day 30 of gestation. The concentration of PAG increase steadly from day 20 to day 30.

Table 1. PAG concentrations in pregnant and non-pregnant ewes from Day 18 to 30 of gestation

	Pregnant ewes (n = 17)		Non-pregnant ewes (n = 6)	
Days	**Mean (ng/mL)**	**SD**	**Mean (ng/mL)**	**SD**
18	0.91	0.51	0.54	0.22
20	1.06	0.48	0.57	0.17
22	1.43	0.48	0.74	0.17
24	1.73	0.50	0.57	0.13
26	2.05	0.34	0.71	0.18
28	2.22	0.29	0.86	0.35
30	2.45	0.36	0.19	0.13

The early pregnancy diagnosis in sheep is essential for good management and especially for planning reproduction of empty ewes and preventing long unproductive periods. Furthermore, it has a commercial interest as it allows not to send pregnant ewes to slaughter or to sale. Early sale of non-pregnant ewes and thereby reducing the feed costs during winter makes early pregnancy diagnosis an economically interesting issue. Early detection of the number of lambs during gestation allows sheep breeders to divide ewes in 2 feeding groups according to their expected litter size. In this way birth weights, weaning weights and survival rates of the lambs are optimized, dystocia and pregnancy toxaemia are prevented.

Alabart *et al.* (2010) showed that in Aragonesa breed, on day 18 and 19 of gestation only 18.8% and 62.1% of pregnant sheep showed PAG levels above 0.8 ng/ml. In Sardinian sheep it has been shown that PAG can be measured in 60.5% on day 18 of gestation. When using an RIA system based on a mixture of different antisera from goat and sheep the accuracy to detect pregnant ewes on day 18 days was 95.3% (Barbato *et al.,* 2009). However, in our work, only from day

24, all pregnant females showed PAGs level above 1.4 ng/ml. The pregnancy diagnosis through PAG determination is a method that has shown its efficiency in both meat and milk breeds (Suffolk, Texel; Assaf, Lacaune) (Vandaele *et al.,* 2005; El Amiri *et al.,* 2007) with differences between single and double pregnancies (Ranilla *et al.,* 1997).

2. Pregnancy monitoring

The PAG concentrations were detected by both ELISA and RIA systems from the 3rd week of gestation in all ewes carying a single foetus (n = 8) and multiple foetus (n = 12) (Fig. 1). The PAG levels increased and decreased depending on the week of gestation and were significantly different from week to week.

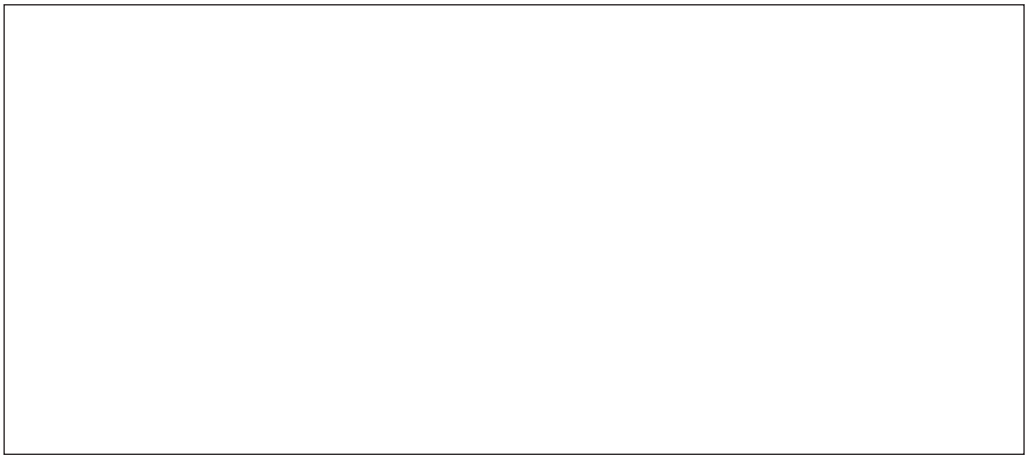

Fig. 1. Plasma concentrations of PAG in single –♦– and multiple –■– pregnancies from the first week of breeding till the 4th week after lambing. A) Profiles of Boujaâd and Boujaâd x D'man sheep based on ELISA technique. B) Profiles of Boujaâd and Boujaâd x D'man sheep based on RIA technique.

In both systems (RIA *vs* ELISA), the PAG concentrations were significantly lower in Boujaâd sheep with single pregnancies than in Boujaâd X D'man sheep with multiple pregnancies. Furthermore, the concentrations in RIA were 3 fold higher than those in ELISA. This observation is clear even the Figs 1 A and B are not plotted in the same scale. The concentrations decreased rapidly after lambing (21 weeks), reaching basal values at fourth week pospartum.

The present study describes for the first time the use of an ELISA for PAG determination for Moroccan sheep pregnancy monitoring. Several earlier studies agreed on the fact that different parameters such as time of gestation, litter size, foetal mass, placentome mass or size, age of ewe, and breed of the foetus may affect PAG concentrations during gestation (Ranilla *et al.,* 1994; Ranilla *et al.,* 1997). However, in our study the only parameters that were significant are the week of gestation ($P < 0.001$ to 0.0001 from week 3 to week 21); the litter size ($P < 0.0001$) and the sex of the foetus ($P < 0.009$). For this later parameter the PAG concentrations were 39.8 ± 4.6, 36.5 ± 3.6 and 22.7 ± 4.1 ng/ml respectively for males, females and both during multiple pregnancies. It is known that sex of lambs can result in a different placental mass, since male foetuses have a higher birth weight and likewise placental weight than female foetuses. Our finding didn't concord with those of Vandeale *et al.* (2005) who did not find any difference between ewes carrying male or female foetuses, possibly because they only explored early stage of gestation and they used an homologous RIA system.

Higher PAG concentrations in twins compared with singles were described in a small number of ewes and cows, and were possibly caused by the higher number of attachment points and thus enhanced synthetic activity of twin placentas. Besides the higher foetal weight and likewise placental mass, the larger foetal-maternal contact surface may be the reason for higher PAG concentrations found in ewes carrying multiples in this study. In addition, the number of the cotyledons was proved to be increased with increasing litter size.

IV – Conclusions

In conclusion, the plasma PAG-ELISA investigated in the present study showed that this technique is proved to be a convenient and reliable means for early pregnancy diagnosis as well as for pregnancy fellow up in sheep. From 24 days of gestation, its reliability achieved 100% and, therefore, matches conventional approaches of pregnancy detection. It also excels some RIA-systems already published.

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