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Original Article

The extent of placental pathology is negatively correlated to birth weight in ewes infected with the wild-type strain of *Chlamydia abortus*

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Abstract

The placenta is the organ that allows the exchange of oxygen and nutrients between maternal and foetal blood, supplying the requirements of the growing foetus. Consequently, any factor that alters placental integrity may affect foetal nutrition, viability and lamb birth weight. Reproductive diseases, such as ovine enzootic abortion (OEA), impact on foetal viability due to the reduction in the functional area for maternofoetal exchange. This study aimed to consider the impact of pathological features of OEA placental lesions on lamb birth weight and indirectly on foetal viability. To investigate the relationship between birth weight and various OEA-related parameters, data from 562 lambs/foetuses from animals experimentally challenged with *Chlamydia abortus* strain S26/3 and from uninfected animals were analysed. The parameters investigated included the number of foetuses/lambs delivered (single/multiple), foetus/lamb sex, length of gestation, the proportion of placentas affected by lesions (percentage of gross placental pathology), foetal viability (live/aborted) and the number of *C. abortus* organisms shed in post-parturition vaginal excretions. The results suggest that the length of gestation and the proportion of placentas affected by lesions are the main contributors to birth weight variability, whereas the other factors, including foetal viability (live or aborted outcomes), were found to be less relevant co-variables. The study determined the strongest positive and negative correlations between birth weight were with the length of gestation and the extent of placental pathology, respectively. These results may indicate that economic losses associated with OEA infections result not only from aborted foetuses but also from the surviving lambs that are born weaker and consequently are more susceptible to diseases.

Keywords:

Ovine Enzootic Abortion; *Chlamydia abortus*; birth weight; placental lesions

Introduction

The placenta is an organ that provides all the nutrients required for foetal growth throughout gestation by transference between maternal and foetal blood. In sheep, there is a positive association between placental weight and lamb birth weight, placental weight and average cotyledon weight and placental efficiency with cotyledon density (Özyürek and Türkyilmaz, 2020; Sen et al., 2013). As the number of caruncles limits the number of cotyledons, each placenta has fewer cotyledons in a multifoetal pregnancy. This limitation in number is compensated for by an increase in the size of each cotyledon (Bell and Ehrhardt, 2002; Bell et al., 1999). This suggests a maternal compensation mechanism operates for adapting to the increasing foetal requirements during gestation. When the increase in surface area of the cotyledons is insufficient and fails to match the foetal nutrient requirements, the placental compensation mechanism fails, causing lower birth weights and, consequently, an increase in lamb death rates (Kaulfuss et al., 2000). Therefore, any factor that affects placental development can also affect foetal viability and birth weight. For example, smoking and diseases such as diabetes mellitus that cause stromal vascular lesions negatively affect birth weight in humans (Ganer Herman et al., 2017; Godfrey et al., 1991), as do infections caused by pathogens targeting the reproductive tissues.

One such pathogen affecting placental sufficiency and development is *Chlamydia abortus*, an obligate intracellular bacterium that is responsible for causing ovine enzootic abortion (OEA). OEA typically causes necrotising suppurative placentitis with thrombosis (Buxton et al., 2002; Longbottom and Coulter, 2003). These lesions cause a decrease in the efficiency of the maternofoetal exchange resulting from epithelial necrosis, oedema, and inflammation that increase placental thickness, the interhaemal distance between maternal and foetal blood and affects the blood supply (Hafez et al., 2010). These pathological changes may impair nutritional and gaseous transplacental exchange (Essig and Longbottom, 2015; Sammin et al., 2009), reducing the functionally effective cotyledonary area. This reduction might affect the nutritional provision of maternal sources to meet foetal demands, reducing foetal vitality and compromising pregnancy (Shukla et al., 2014).

The pathological lesions associated with OEA appear during the later stages of gestation, when the foetal demands are higher (Reynolds et al., 2010). Although OEA lesions may result in lower birth weight, such an association has not been previously published. This study aims to evaluate the impact of different characteristics typically associated with OEA infections with the birth weight of lambs delivered from infected ewes.

Materials and methods

OEA and birth weight of lambs

Data used for these analyses were obtained from ewes that were experimentally challenged with *C. abortus* strain S26/3, which served as positive control animals, and from uninfected negative control ewes, in studies carried out at the Moredun Research Institute (MRI) between 2009 and 2018 (Livingstone et al., 2021; Livingstone et al., 2017; Livingstone et al., 2009; Longbottom et al., 2013). These challenge experiments were performed as part of various pathogenesis (Livingstone et al., 2017; Livingstone et al., 2009; Longbottom et al., 2013) and vaccine efficacy (Livingstone et al., 2021) studies. In total, data from 943 lambs/foetuses from 562 ewes (n=346 ewes and n=563 lambs/foetuses from experimentally infected animals and n=216 ewes and n=380 lambs/foetuses from uninfected/negative control animals) were analysed. All animals were Scottish Blackface or Scotch Mule (crossbred sheep of Scottish Blackface ewes sired by Bluefaced Leicester rams) sheep (aged 3–6 years) obtained from OEA-free flocks and screened by rOMP90-3 indirect ELISA enzyme-linked immunosorbent assay (ELISA) to ensure all animals were seronegative for *C. abortus* (Wilson et al., 2009). All challenge animals were inoculated on day 70 of gestation with 2 mL of inoculum containing 2 x 10^6 inclusion forming units of *C. abortus* strain S26/3 administered by subcutaneous injection over the left precrural lymph node, as previously described (Livingstone et al., 2021; Livingstone et al., 2017; Livingstone et al., 2009; Longbottom et al., 2013).

In each of these previous studies, the placentas were collected, tagged, photographed and examined to determine the extent of visible OEA gross pathological lesions (proportion of placentas affected estimated as a percentage), as previously described (Buxton et al., 2002; Stamp et al., 1950). Additional features that could also be associated with differences in birth weight were recorded. These included: foetal sex (male/female); number of foetuses/lambs (singles/twins/triplets/quadruplets); length of gestation (days); viability of the foetus (live/aborted outcomes); the presence of pathogen in placental smears (assessed following chemical staining with modified Ziehl-Neelsen (mZN)); and the amount of pathogen shed post parturition) estimated from cervicovaginal mucus (CVM) collected from vaginal swabs (measured by quantitative real-time PCR (qPCR) based on the *ompA* gene). The mZN scoring ('0' for no; '1' for low; '2' for moderate; and '3' for high numbers of *C. abortus* elementary bodies (EBs) observed under high-power microscopy) and presented in Table 1 as the mean of the scores. qPCR and mZN analyses were the same for all the studies and were performed as previously described (Livingstone et al., 2009) by the same person with over 20 years of laboratory expertise in conducting these analyses.

Statistical methods

The associations between lamb birth weight as the response variable and the proportion of the placenta affected, days of gestation, litter size sex of foetus/lamb, qualitative estimate of pathogen in placental smears (mZN score), abortion outcome and quantitative estimate of pathogen shed at parturition (qPCR load) as explanatory variables were investigated by fitting a regression model by generalised least squares (GLS), maximising the restricted log-likelihood (REML) to allow for a non-homoscedastic variance structure. The statistical significance of the individual terms of the model was assessed by F-tests (conclusions based on the common 5% significance level). This was accompanied by a variable importance analysis using an information-theory approach. Namely, the relative importance of the explanatory variables was assessed by sequentially considering all combinations (subsets) of variables and comparing the resulting models by the Akaike information criterion (AIC) (Burnham and Anderson, 2002). This allowed the explanatory variables to be ranked according to their relative importance based on the sum of Akaike weights over all possible models (values in [0, 1] from less to more importance), derived from the original full model in which a variable was included (Burnham and Anderson, 2002). Finally, we built on the information provided by the previous analyses to estimate a partial correlation network of the quantitative variables. This was useful to visualise the sign and strength of their pairwise relationships while filtering out the influence of the remaining variables. Using Spearman's partial correlations allowed us to relax the ordinary assumption of normally distributed variables.

All the statistical analyses were performed on the R system for statistical computing v4 (R Core Team, 2021).

Results

Birth weight and OEA-related *parameters*

Figure 1 depicts the typical range of placental lesions (highlighted in the figures by a yellow outline) observed for sheep that aborted (panels D to F), produced a mix of live and aborted lambs (C) or produced apparently normal live lambs (panel B). These show the typical large range of lesions (20-100%) observed in placentas from sheep that abort their lambs. Figure 2 shows foetuses (panels A, B and C) and lambs (panels D, E, F) that were typically observed during the trial. The aborted foetuses (Fig 2 A, B) showed less foetal development with an absence of wool covering, asymmetric proportion of the body parts, with long legs and small head which had incomplete remodelling. In contrast, Fig 2 C shows a preterm foetus that is almost fully developed except for a small distortion of the upper maxillary. Fig 2 D, E and F show full term lambs with complete development of their morphology and wool covering that were euthanised between 24 and 48 hs after lambing. In Fig 2E, the upper lamb, which was larger and heavier, was normal and was euthanised 24 hs after lambing, while the lower lamb was a stillbirth (died shortly after parturition) and was clearly smaller in size and weight.

The data from lambs/foetuses from the experimental challenge trials were sorted according to birth weight for analysis to determine associations with the various OEA-related parameters (Table 1). The results clearly show that the birth weight of lambs/foetuses is positively associated with the length of gestation and negatively associated with the proportion of placentas affected by lesions, estimate of the amount of the pathogen in placental smears (mZN score) and the amount of *C. abortus* shed at parturition (qPCR results). Lambing outcomes (dead foetuses or live lambs) (Figure 2) were also found to be associated with birth weight, this association being positive for the delivery of live lambs and negative with the delivery of dead foetuses (Table 1).

Regression analysis

Table 2 summarises estimates from the GLS regression model fitted to birth weight and proportion of the placenta affected by lesions, days of gestation, litter size (single or multiple births/abortions), sex of foetus/lamb, bacterial load in placental smears (mZN score), abortion outcome and an estimate of the number of *C. abortus* organisms shed post-parturition (qPCR).

The results suggest that all the explanatory variables, except the type of gestation and number of *C. abortus* genomic copies determined by qPCR, have a statistically significant association with birth weight variability (Table 2). Analysis based on Akaike weights, shows that the length of gestation, proportion of placenta affected by lesions and foetal sex are the main contributors to birth weight variability, whereas mZN score and foetal viability (live or aborted) are the least relevant ones.

Correlation network analysis

Figure 3 shows the estimated Spearman's partial correlation network between quantitative variables. Focusing on correlations with birth weight (WgK in Fig 3), the strongest positive correlation was observed with days of gestation (DG in Fig 3) (+0.25), while the greatest negative correlation was with proportion of the placenta affected by lesions (PrL in Fig 3) (-

0.18). However, the strongest associations were not linked to birth weight, instead they were observed between the proportion of affected placenta and placental pathogen load (mZN in Fig 3) (+0.77) and between mZN pathogen load and pathogen shedding loads (qPCR in Fig 3) (+0.36).

Discussion

The purpose of this study was to analyse how different factors associated with OEA infection correlate with the foetal/lamb birth weight with *C. abortus* infected placentas showing differences in severity of lesions. Previous studies have shown that a direct correlation occurs between birth weight and physiological features including placental weight, placental blood flow and vascularity, number or size of cotyledons and birth weight of the lambs (Bleker et al., 2006; Dwyer et al., 2005; Hafez et al., 2010; Ocak et al., 2009; Özyürek and Türkyilmaz, 2020). In contrast, the goal of this study was to consider some pathological features rather than solely physiological parameters allowing the impact of OEA-associated placental lesions on birth weight and indirectly on foetal viability to be described.

The present study analysed the data obtained from four experimental *C. abortus* challenge studies performed at MRI. These studies were assessed under controlled conditions, which allowed us to obtain complete and detailed information for the substantial number of cases included in the analyses. To avoid any interference of the trial experimental data in these studies, we only included data from the control group animals (negative and positive challenge animals). As a result of these analyses, it is evident that gross placental pathology is inversely associated with birth weight. This result agrees with the fact that the placenta is the organ that provides all the nutrients required by the foetus; therefore, anything that negatively affects the placenta will consequently reduce foetal nutrition and the weight of the developing foetus. Indeed, a positive correlation between average cotyledon surface area and the weight of delivered lambs has been reported (Özyürek and Türkyilmaz, 2020). Considering this, it is logical that diseases such as OEA, which cause placentitis, may affect nutritional placental sufficiency and hence, lead to lower lamb birth weights. Lower birth weights are associated with poorer lamb viability (Gardner et al., 2007; Mukasa-Mugerwa et al., 1994). However, the longer-term viability of lambs (for example 'survival to weaning') born to infected mothers was not an objective of this study as the animals were controls from various pathogenesis and vaccine efficacy studies, which had their own specific experimental designs and objectives. Nevertheless, 'survival to weaning' would be an interesting factor that could be added to the analyses presented here in a future study.

Although the gross placental lesions have an impact on birth weight, in this study, other co-variables including the length of gestation also greatly influenced lamb weight. In OEA, placental lesions become more prominent in late gestation when there is normally an increase in foetal growth rate (Caton et al., 2009; Redmer et al., 2004; Vonnahme et al., 2003). These lesions, which as stated above, reduce placental sufficiency and foetal development, resulting in pre-term death of the foetus *in utero* and expulsion of the foetus earlier than expected or the premature delivery of lambs with a lower birth weight. This is also evident in the influence that the foetal/lamb viability (p=0.0214) showed on the birth weight (Table 2). These findings agree with previous reports where the birth weight of the lambs that were born from non-infected ewes was significantly higher than those delivered earlier in gestation in ewes challenged with *C. abortus* strain AB7 (Garcia-Seco et al., 2016).

Other variables such as litter size or placental smear load (mZN scores) and post-parturition shedding (qPCR results) show comparable proportions within the weight intervals (Table 1), suggesting no significant association with birth weight. Foetal sex did not differ with weight except in the highest weight interval where the percentage of males was higher than females. Birth weight aside, other strong correlations between variables were found, e.g., between PrL and mZN, between mZN and qPCR, and between DG and PrL (Figure 1). The strong relationship between PrL and mZN may be due to the OEA placental lesions being the result of chlamydial growth, which produces necrosis when EBs are released following the lysis of the host cell. Consistent with this, the correlation of mZN and qPCR may indicate that when the infected placenta (detected in this case by mZN) passes throughout the cervix, some chlamydial organisms may remain in the genital tract and can subsequently be detected on the CVM-swab by qPCR. On the other hand, the negative correlation between PrL and DG could indicate that when the lesions are more severe, the possibility of premature parturition or abortion is also higher, causing a reduction in the length of the gestation and death of the foetus.

Conclusions

This study provides objective evidence of a negative association between OEA lesions and birth weight. Additionally, these reductions in birth weight in live lambs cause tangible economic losses in addition to the ones related to OEA abortions. Combined analysis of the different factors associated with OEA used in this study allowed the relative impact of these variables and how they are related to be defined. This study provides additional evidence of a wider economic impact of OEA, increasing our knowledge of the pathogenesis of the disease.

Conflict of interest statement

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1 Descriptive summary of data obtained sorted by weight interval quartiles (cut-offs 25%, 50%, 75%) including foetus/lambs from both, challenged and uninfected ewes.

Foetus/ Lamb Weight intervals	Number of lambs / foetuses	Placental lesion (%)*	Sex (%)		Litter size (%)		Length of gestation (days)*	Lambing outcome (%)		Placental smears (mZN)*[b]	Shedding (qPCR)[c]
			Female	Male	Single	Multiple		Live	Dead		
[0.9,3.5]	236	17.1 (1.2)	53.9	46.2	23.1	76.9	138 (1)	51.8	48.2	2.5 (1.1)	92313 (1.4)
[3.5,4.2]	236	2.5 (1.1)	53.0	47.0	17.0	83.0	144.0 (1)	90.7	9.3	1.4 (1.0)	1858.9 (1.4)
[4.2,5.0]	236	1.4 (1.1)	53.5	46.5	22.1	77.9	145.3 (1)	98.6	1.4	1.2 (1.0)	417. (1.3)
[5.0,7.6]	235	1.1 (1.0)	43.5	56.5	22.5	77.5	146.6 (1)	99.7	0.3	1.1 (1.0)	174.4 (1.2)

*Values are geometric means (geometric standard error of the mean in parenthesis)
[a] Intervals are set by quartiles

[b] Estimation of of *C. abortus* EBs observed, under high-power microscopy, in mZN-stained placental smears (Negative. 0, occasional numbers of EBs, ‘1’; low, ‘2’; moderate, ‘3’; high, ‘4’) (Livingstone et al., 2009)

[c] Number of *C. abortus* genome copies per µL of vaginal swab total extracted DNA, as determined by qPCR

Table 2 Regression analysis of birth weight of foetuses/lambs from both, challenged and uninfected ewes.

	***F*-value**	***p*-value**	***Sum Akaike weights***
(Intercept)	20988.22	<0.0001	
Length of gestation[a]	1077.153	<0.0001	1.00
Litter size[b]	1.07	0.3012	0.27
Foetal sex	11.694	0.0007	1.00
Placental lesions[c]	108.523	<0.0001	1.00
Placental smears[d]	4.276	0.0389	0.72

Foetal viability[e]	5.313	0.0214	0.85
Organism shedding[f]	2.691	0.1013	0.58

[a] In days
[b] Single, twin, triplet, or quadruplet
[c] Proportion (%) of the placenta affected by gross lesions (Buxton et al., 2002)
[d] Placental pathogen scores following staining with modified Ziehl-Neelsen (Livingstone et al., 2009)
[e] Born live or aborted
[f] Estimate of pathogen load (genomic copies per µL of total extracted DNA) in vaginal swabs (Caspe et al., 2020; Livingstone et al., 2009)

Figure legends

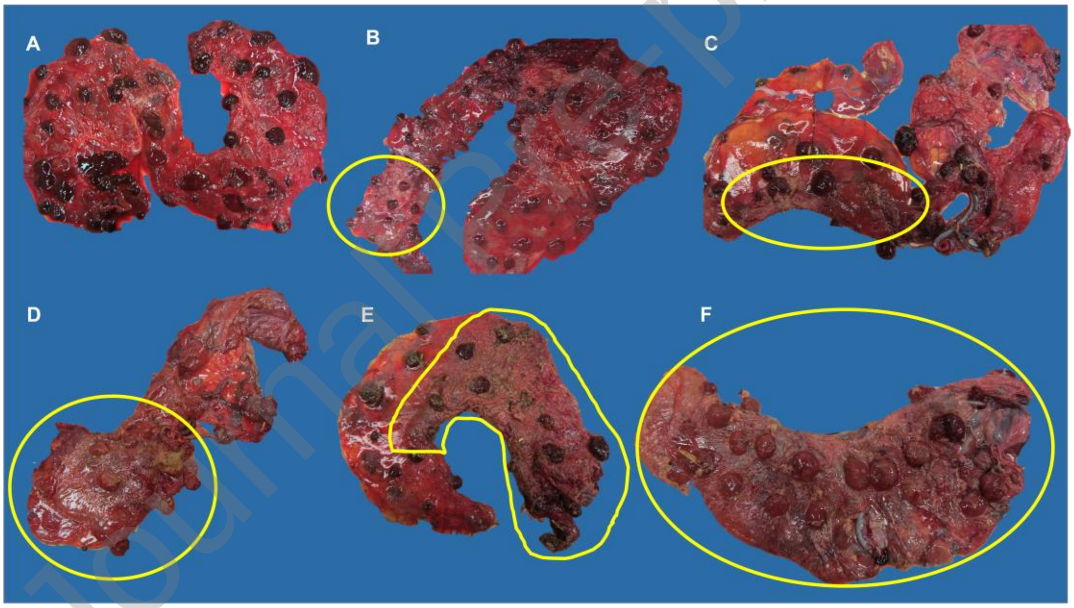

Fig.1. Placentas showing the range of pathological lesions observed. Note the differences in the proportion of each placenta affected, evident as the presence of necrotic debris covering the surface (area of lesion outlined in yellow): 0 % (A); 5% (B); 20% (C); 50% (D); 70% (E); and 100% (F).

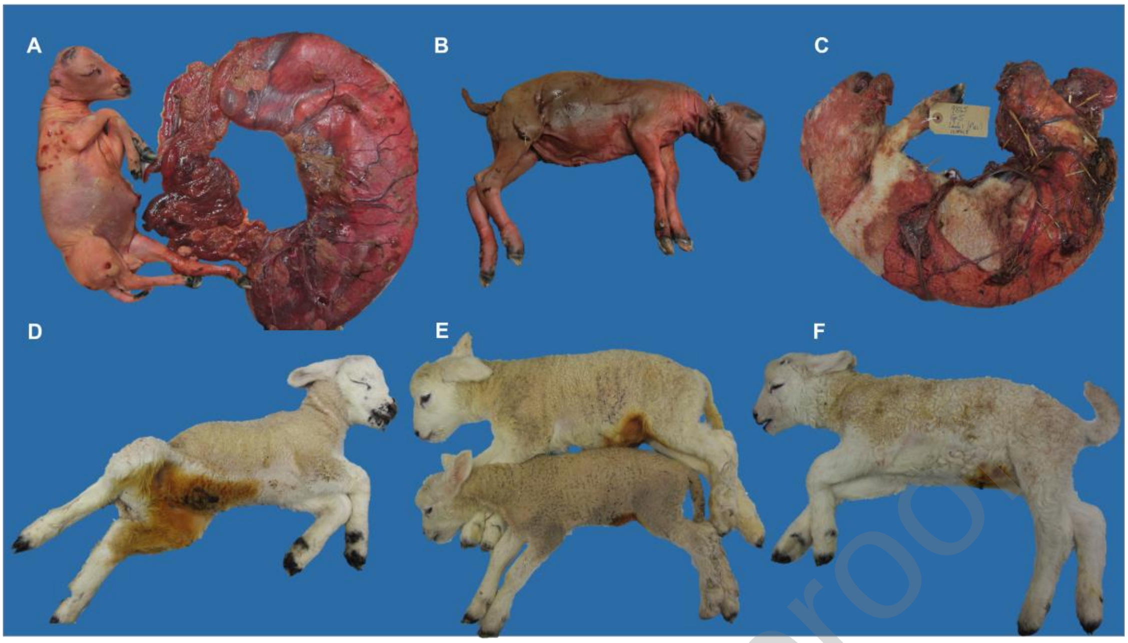

Fig.2. Different pregnancy outcomes were observed during the studies. Note differences in foetal maturity and development between aborted foetuses (A. B and C; absence of wool coats (only in A, B), remodeling of head structure, relatively small size and low birth weight, and poor muscle development), stillbirth (E-bottom) and lambs born alive (D, E-top, and F) (euthanised 24 hs after lambing). (A) Two foetuses aborted at 121 days of gestation (dg) from the same ewe (on the left: 2 kg, on right still inside placenta:1.7 kg); (B) foetus aborted at 124 dg (1.9 kg); (C) foetus aborted close to expected parturition at 135 dg (4kg); (D) live lamb born at 140 dg; (E) two live lambs born at 140 dg from the same ewe, one live (top:4 kg) and one stillborn (bottom: 2.75 kg); (A, B, C, D and E are foetuses/lambs born from challenge ewes); and (F) a live lamb born at 147 dg (uninfected; 4.8 kg).

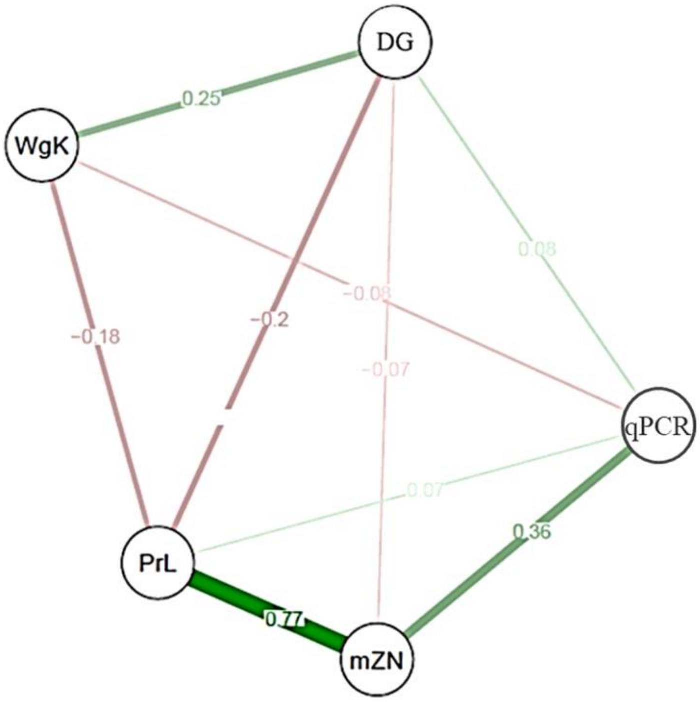

Fig.3. Partial correlation network. The graph shows the positive (green) and negative (red) associations between the analysed variables, while the thickness of the lines shows the strength of the correlation (thicker lines correspond to a stronger association). Note the strongest positive correlation between percentage placental lesions (PrL) and placental mZN score (mZN), between mZN and extent of organism shedding at parturition (qPCR) and between birth weight (WgK) and days of gestation (DG). Negative correlations were observed between WgK and PrL and between PrL and DG.

Declaration of Competing Interest

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the paper's content.

Highlights (for review)

- Ovine enzootic abortion (OEA) infections cause economic losses in ovine flocks.
- Aborted foetuses and weak lambs are the cause of production losses in OEA.
- Lambs born to affected ewes are weaker and more susceptible to disease.
- The degree of the placental lesions is associated with the gestation length and, consequently, with foetal viability.
- First report of relationship between the placental lesion and disease outcome.