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RESEARCH ARTICLE

# Relationship between Pro-Inflammatory Cytokines, IL-10 Anti-Inflammatory Cytokine and Serum Proteins in Healthy Lambs and with Diarrhea

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**ABSTRACT**

We determined the levels of the cytokines IL-6, IL-1β, IFN-γ and IL-10 in fifteen lambs during the first month of life. The correlation between these cytokines and serum protein fractions was studied. In addition, the concentration of these cytokines in fourteen lambs with diarrhea was determined. IFN-γ (73.85±121 pg/ml) peaked at day 1, IL-6 (7910±2329 pg/ml) and IL-1β (5234±2461 pg/ml) peaked at day 4, and IL-10 (63.4±5.2 pg/ml), peaked at day 28. At day 11 the highest correlation between IL-6 and IL-1β was identified. An increase ($P<0.01$) in IL-1β concentration was detected in diarrheic lambs. There was a positive correlation between IL-6, IL-1β and total protein levels in sick lambs and also between IL-6 and total globulins and IL-1β and total globulins. These results indicate that with the exception of IL-10, cytokine concentrations in healthy lambs follow time-dependent evolution. The higher concentration of IL-1β detected in diarrheic lambs could be used as are reliable inflammatory marker in ovine pathology. Further work is required to understand the role of cytokines in both physiological and pathological processes.



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## INTRODUCTION

Cytokines are a group of soluble proteins that, at very low concentrations, are able to regulate the function of cells and tissues. They have different functions and have a short half-life, are locally produced in response to stimuli and may act in an autocrine, exocrine, or endocrine fashion (Balaram *et al.,* 2009; Khalid *et al*., 2015). Th1 cells produce IFN-gamma (IFN-γ). This cytokine activates macrophages to destroy ingested microorganisms (Tizard, 2013). IL-1β is a pro-inflammatory cytokine which is secreted primarily by activated monocytes. Clinically, IL-1β contributes to the defense response against pathogens by generating fever and promoting the influx of leukocytes into the sites of infection (Tizard, 2013). Another pro-inflammatory cytokine secreted by T cells and macrophages is IL-6, a potent pyrogen acting through receptors located in hepatocytes to produce acute phase proteins (APP) (Petersen *et al.,* 2004). IL-10, an anti-inflammatory cytokine, is a major antagonist of the Th1 type response (Balaram *et al.,* 2009).

The identification and measurement factors, such as cytokines, that regulate immune responses are important in understanding the pathogenesis of the diseases, as well as for diagnosis and control. Cytokines are also implicated in the pathogenesis of multiple processes in ovine diseases (Scheerlinck and Yen 2005; Kabaroff *et al.,* 2006). In bovine, inflammatory cytokines have been utilized as a diagnostic aid, mainly for mastitis (Hagiwara *et al.,* 2001; Sakemi *et al.,* 2011). Levels of cytokines measured in serum can be used to assess the innate immune system response to infection or inflammation. In equine, Pusterla *et al.* (2006) and Burton *et al.* (2009) used serum cytokine concentrations as molecular markers of sepsis in neonatal foals, considering it to be of diagnostic value.

To our knowledge there is no research demonstrating cytokine profiles in the serum of healthy or diarrheic lambs during the first month of life. Analysis could assist in the diagnosis and increase our knowledge and understanding of the pathogenesis of inflammatory responses in ovine diseases, especially under subclinical conditions. We hypothesized that cytokines levels would increase in sick lambs and these animals would exhibit cytokine levels distinctive to those detected in healthy animals. The authors also hypothesized that in serum, cytokine concentration would correlate with the concentration of protein fractions.

## MATERIALS AND METHODS

**Animals:** The Bioethical Committee of Zaragoza University approved all procedures. Fifteen Rasa Aragonesa lambs were selected from a commercial sheep farm. All lambs, with the exception of one animal, were born to twin-bearing ewes. Delivery was natural with no medication or human intervention required. The lambs were immediately removed from the dams, their umbilical cord disinfected, and injected with a dose of vitamin.

To assess and monitor clinical status of the animals, nine male and six female lambs, were observed twice daily during the experimental period with a view to identifying any clinical signs of disease. The BW at birth were between 2 and 4.5 kg.

Blood samples (4 ml) were collected from the jugular vein into clot activator vacutainer tubes. Samples were obtained at birth, just before suckling colostrum and on days 1, 4, 11, 18 and 28. Fourteen Rasa Aragonesa sick lambs from two different farms with the same management as the healthy lambs were selected. Acute diarrheic lambs were selected based on clinical signs of dirty hocks and tails. The animals did not receive any antibiotic treatment before blood sampling. Blood sample (4 ml) was obtained from the jugular vein and sera separated in the laboratory. The mean age of these lambs was 11 (farm A, n=7) and 18 days old (farm B, n=7).

Bacteriological testing to diagnose the cause of the diarrhea was performed on rectal swabs taken from six lambs. The microorganisms isolated from the diarrheic lambs were *E. coli, Cl. perfringens, Campylobacter* spp, *Cryptosporidium parvum* and *Eimeria* spp.

**Analysis of cytokines in serum samples:** IL-1β and IL-6 were performed using a commercial ELISA kit (Nova Tein Bio Inc. Cambridge, USA), following the manufacture's recommendations. Ovine IFN-γ was detected with a bovine ELISA kit from Mab Tech (Cincinnati, USA), which has good cross-reactivity with sheep IFN-γ. Sheep IL-10 was determined by a competitive ELISA kit from Cusabio Biotech (Wuhan, China). The optical density within the plate wells was determined at 450 nm in a Labsystem Multiskan RC microplate reader (Vantaa, Finland). All samples were assayed in duplicate. Concentration of cytokines in sera was determined from plotting a standard curve as provided in the kit.

**Serum protein electrophoresis:** Serum protein fractions were segregated by electrophoresis with a Hydrogel 7 kit (Sebia Hispania, Barcelona, Spain) and were read with a Shimadzu CS-9000 photodensitometer (Kyoto, Japan). Albumin, alpha-, beta- and gamma-globulins were calculated. Serum total protein was analysed with an ACE® autoanalyser (nº AE5-23, Clinical Chemistry System, Alfa-Wassermann, USA) using the manufacturer's reagent and recommended procedures.

**Statistical analysis:** The data were analyzed using StatView 5.0.1 software. Differences between healthy and sick lambs for different cytokines and serum concentration, at six time points, were analyzed. Paired analysis was performed utilizing Wilcoxon signed rank test and unpaired with the Mann-Whitney U-test. The correlation between the cytokines and also the cytokines and serum protein fractions were determined with a Spearman rank correlation test. In order to assess the relationship between each cytokine level, the BW was differentiated into two categories: low weight (2-3 kg BW n=5) and normal weight (>3 kg BW, n=10). In addition the sex of each lamb was recorded in order to identify any gender-related differences in their cytokine concentrations. The level of significance was set at $P<0.05$.

## RESULTS

**Cytokine concentrations during the first month of life in healthy lambs:** The results are shown in Fig. 1. At birth none of the four cytokines were detected in the sera of the neonates but became detectable on the first day after receiving colostrum. The higher concentration was reached at day 1 for IFN-γ (73.85±121 pg/ml), but no statistically significant difference ($P>0.05$) was determined between the days. IL-6 (7910±2329 pg/ml) and IL-1β (5234±2461 pg/ml) peaked at day 4, subsequently concentrations gradually decreased until the last day of analysis. However, the serum concentration of IL-10 increased progressively over time and the concentration peaked at day 28 (63.4±5.2 pg/ml).

**Effect of gender and BW on cytokine concentrations:** There was no statistically significant difference in the cytokine concentrations detected between the sexes throughout the study period (data not show). The cytokine level was higher in the heavier lambs than that determined the lighter weight animals at birth. However, in normal and low BW lambs at birth a statistically significant difference ($P<0.05$) for IL-1β, IL-6 and IFN-γ cytokine levels were detected (Fig. 2) at day 18.

**Clinical status and serum protein levels:** Table 1 shows the evolution of serum protein levels in lambs. The higher level of total proteins and γ-globulins were found at day 4, followed by a gradual decrease in concentration until the end of the study. No effect from gender or BW was noted ($P>0.05$).

**Correlation between cytokines and serum protein fractions in healthy lambs:** A significantly positive correlation was detected between cytokines and serum protein fractions in healthy lambs, as shown inTable2. We determined positive and significant correlations between IL-6 and IL-1β ($P<0.05$) at days 4, 11 and 18, with the greatest correlation identified at day 11. Similarly, positive correlations ($P<0.05$) were observed between IL-1β and total proteins, alpha, γ-globulins and total globulins. Also, statistically significant differences ($P<0.05$) were found between IL-6 and total proteins, γ-globulins and total globulins.

**Cytokine concentrations in sick lambs:** Figure 3 shows a comparison of cytokine concentrations in sick and healthy lambs. Only a statistically significant difference ($P<0.05$) was detected for IL-1β for the two days analyzed, with the levels in sick lambs higher than those in healthy animals.

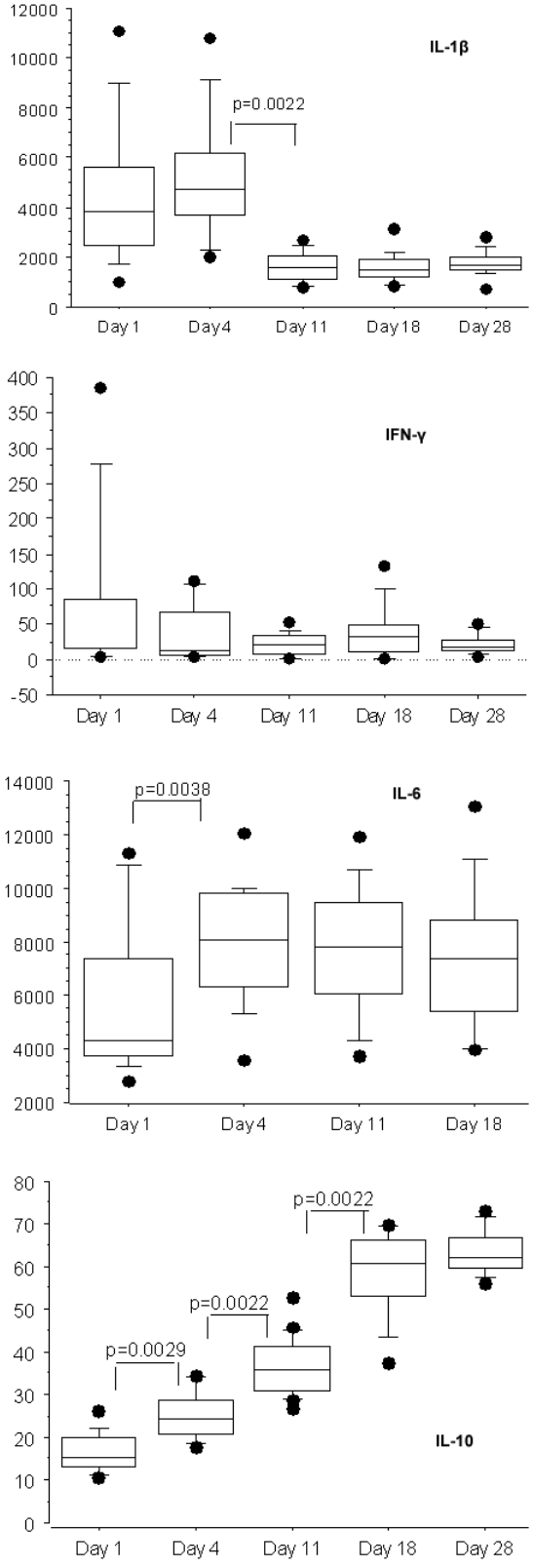


**Fig. 1:** Cytokine levels of IL-1β, IL-6, IFN-γ and IL-10 in newborn lambs during the first month of life. Cytokine concentrations are expressed as pg/ml. Note: for IL-6 only up to day 18 was analyzed. Results are shown as Box and Whisker diagram. Box represents the 25th and 75th percentile, horizontal line represents the median, whiskers represent the 10th and 90th percentiles and plot points represent the range. Statistically significant differences between days were analyzed by the Mann-Whitney U test (n=15).

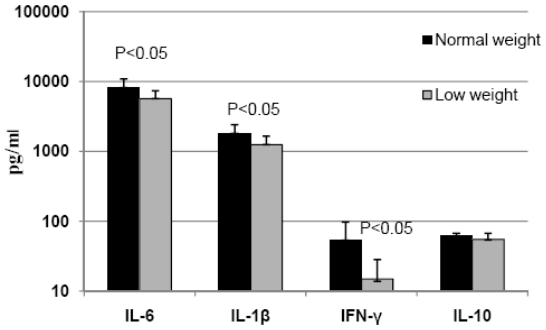


**Fig. 2:** Statistically significant differences in cytokine concentrations at 18 days old between normal (>3 kg) and low (2-3 kg) BW, healthy lambs. Each bar represents the mean of the normal (n=10) and low BW lambs (n=5). Values are mean and SD in logarithmic scale.

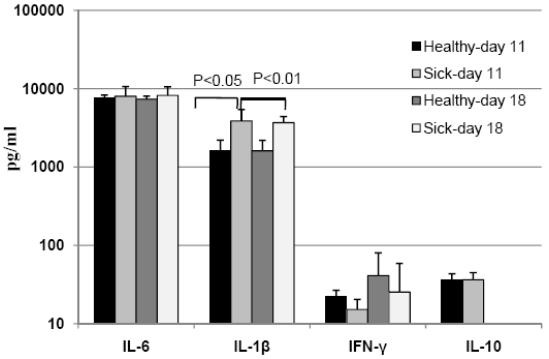


**Fig. 3:** Statistically significant differences in cytokine concentrations between healthy (n=15) and sick lambs in at day 11 (n=7) and 18 (n=7). For IL-10, serum from 11 day old lambs was analyzed. Values are mean and SD in logarithmic scale.

**Correlation between cytokines and serum protein fractions in sick lambs:** Table 3 shows results obtained from Spearman rank correlation analysis of data from sick lambs. Positive correlations (P<0.01) between IL-6 and IL-1β and for IL-6 and total proteins, γ-globulins and total globulins (P<0.05) were detected. Moreover, there were positive correlations (P<0.05) between IL-1β and total globulins and total proteins.

## DISCUSSION

The aim of this study was to determine the evolution of the serum levels of pro-inflammatory cytokines (IL-1β and IL-6), Th1 cytokine (IFN-γ) and anti-inflammatory Th2 cytokine (IL-10) in lambs during the first month of life and whether they could be utilized as biomarkers for disease such as diarrhea. Cytokines have not been as intensely evaluated in sheep as they have in other species. IL-6 and IL-10 levels have been analyzed in healthy and sick foals (Pusterla *et al.*, 2006; Burton *et al.*, 2009) in order to improve the diagnosis and evaluation of many diseases. In the same way, IL-1β, IL-6 and IFN-γ were studied in calves (Hagiwara *et al.*, 2001; Yamanaka *et al.*, 2003) and seven cytokines were analyzed in suckling piglets (Nguyen *et al.*, 2007). Thus far the authors have not found any reference regarding the measurement of serum cytokines in neonatal lambs by ELISA and this is the first paper to reveal these values. This study provides novel data on the proteins that could be used for evaluating the pathogenesis of inflammatory diseases in the ovine species.

**Table 1:** Serum protein electrophoresis (g/dl) in healthy lambs during the first month of life (n=15) and in sick lambs at 11 to 18 days of life (n=14). Data are mean ±SD.

| | Day 0 | Day 4 | Day 11 | Day 18 | Day 28 | Sick |
|---|---|---|---|---|---|---|
| | | | Healthy | | | |
| Total protein | 4.53±0.82 | 6.83±0.98 | 5.64±0.68 | 5.52±0.51 | 5.43±0.69 | 6.03±1.7 |
| Albumin | 2.86±0.48 | 2.88±0.27 | 2.82±0.31 | 3.08±0.27 | 3.24±0.45 | 3.43±0.76 |
| α-globulin | 0.59±0.33 | 0.64±0.27 | 0.51±0.19 | 0.86±0.26 | 0.41±0.16 | 0.41±0.24 |
| β-globulin | 0.80±0.47 | 0.71±0.16 | 1.12±1.0 | 0.86±0.26 | 0.96±0.36 | 1.15±0.46 |
| γ-globulin | 0.28±0.4 | 2.63±1.1 | 1.23±0.61 | 1.16±0.52 | 0.85±0.35 | 1.07±1.1 |
| Total globulin | 1.67±0.49 | 3.99±0.99 | 2.86±0.71 | 2.44±0.37 | 2.22±0.49 | 2.59±1.17 |
| A/G ratio | 1.81±0.43 | 0.77±0.21 | 1.04±0.27 | 1.29±0.21 | 1.52±0.34 | 1.44±0.39 |

A/G ratio=albumin/globulin ratio. Data from day 0 were before colostrum intake

**Table 2:** Positive and significant correlations using Spearman rank correlation coefficient found between IL-6, IL-1β cytokines and proteins fractions in healthy lambs

| Correlation | All days | | Day 4 | | Day 11 | | Day 18 | |
|---|---|---|---|---|---|---|---|---|
| | Rho | P value | Rho | P value | Rho | P value | Rho | P value |
| IL-6/IL-1β | 0.263 | 0.046 | 0.632 | 0.028 | 0.943 | 0.001 | 0.908 | 0.001 |
| IL-6/total proteins | 0.429 | 0.004 | | | 0.682 | 0.041 | | |
| IL-6/γ-globulins | 0.418 | 0.005 | | | | | | |
| IL-6/total globulins | 0.497 | 0.001 | | | | | 0.551 | 0.047 |
| IL-1β/total proteins | 0.273 | 0.043 | | | | | | |
| IL-1β/α-globulins | 0.39 | 0.004 | | | | | | |
| IL-1β/γ-globulins | 0.272 | 0.046 | | | | | | |
| IL-1β/total globulins | 0.360 | 0.008 | | | 0.673 | 0.044 | | |

**Table 3:** Positive and significant correlations using Spearman rank correlation coefficient found between IL-6 and IL-1β cytokines and protein fractions in sick lambs

| Correlation | Rho | P value |
|---|---|---|
| IL-6/IL-1β | 0.776 | 0.010 |
| IL-6/total proteins | 0.772 | 0.008 |
| IL-6/γ-globulins | 0.645 | 0.041 |
| IL-6/total globulins | 0.727 | 0.022 |
| IL-1β/total proteins | 0.601 | 0.047 |
| IL-1β/total globulins | 0.636 | 0.044 |

The four cytokines analyzed were detected at day 1 after birth and the peak levels were reached at day 4. Our results demonstrated that prior to the ingestion of colostrum, serum cytokine levels were undetectable. Therefore, the source of the cytokines in neonates is derived from colostrum. This mammary secretion is very rich in cytokines in sheep, as comparative studies have demonstrated with bovine colostrum (Hagiwara *et al.,* 2001; Yamanaka *et al.,* 2003), mare colostrum (Burton *et al.,* 2009; Secor *et al.,* 2012), sow colostrum (Nguyen *et al.,* 2007) and human colostrum and milk (Agarwal *et al.,* 2011). The evolution of the levels observed in this study is consistent with ingestion of the cytokines from colostrum. No detectable endogenous production of cytokines was recorded until 33 days old in gnotobiotic piglets by Nguyen *et al.* (2007). In a study by Yamanaka *et al.* (2003), serum cytokines in calves were not produced by lymphocytes in newborns. A similar picture of cytokine evolution to that observed in this work was detected in calves, but Yamanaka *et al.* (2003), found that at day 28 levels of IL-1β, IL-6 and IFN-γ were undetectable in the calf serum. The evolution of IL-10 was surprising as a continuous increase in concentration over time was observed. The reason why IL-10 was seen to increase in our lambs is inconclusive. A possibility is that lambs were continuously obtaining IL-10 from their dam's milk and this resulted in the increase in serum cytokine concentration. No decline IL-10 level over time has been described in human breast milk (Agarwal *et al.,* 2011), but the IL-10 concentration in sheep milk was unknown.

Gender had no effect on cytokine concentrations. We did not find consistent data from the veterinary literature to compare to our results. In human medicine there is lack of consistency between studies that investigated cytokine expression with respect to fetal gender. Consequently, Weissenbacher *et al.* (2012) concluded that no significant differences between 15 cytokine levels and fetal gender in amniotic fluid could be found. In addition, BW had no effect on cytokine concentration, except at day 18. We have found no references with which to compare our data, therefore our results are difficult to explain. A possibility could be the differences in cytokine concentrations in the sheep colostrum compared with mature milk, as this difference has been described for human breast milk (Agarwal *et al.,* 2011). Further research is required to address this hypothesis, including the study of premature and normal weight lambs and the analysis of cytokine levels throughout gestation, birth and the lactation period.

Concentrations of the serum proteins were within normal range and were similar to previous observations made in lambs of the same breed and livestock management and are consistent with colostrum intake as reported for lambs (Loste *et al.,* 2008). In this work, we found a positive and significant correlation between the cytokines IL-1β and IL-6 and serum protein fractions in healthy lambs. IL-1β and IL-6 are two pro-inflammatory cytokines that have been detected in many diseases in both human and veterinary medicine (Burton *et al.,* 2009; Hagiwara *et al.,* 2001; Lodha *et al.,* 2010). Cytokines are small, secreted proteins which mediate and regulate immunity, inflammation and hematopoiesis (Balaram *et al.,* 2009), explaining the results found between these two cytokines and the protein profile in lamb's serum. Levels of total proteins and γ-globulins are correlated with colostrum intake and IgG concentration, as demonstrated with small ruminants (Fernández *et al.,* 2006; Loste *et al.,* 2008). On the other hand, APP, induced by cytokines, act as messengers between the local site of injury and the hepatocytes (Petersen *et al.,* 2004), being serum amyloid A (SAA) and C-reactive protein, induced primarily by IL-1 type cytokine; fibrinogen and haptoglobin by IL-6. In this work, α-globulin concentrations were related to IL-1β levels and SSA and haptoglobin are in the α-globulin region

(Eckersall, 2008). There was a correlation between IL-1β and IL-6, two pro-inflammatory cytokines that share many functions. This positive correlation has been intimated in serum from pigs (Barbé *et al.,* 2011). The levels of these cytokines could be used to assess inflammatory processes during the perinatal period in lambs, consistent with those used in foals (Pusterla *et al.,*2006; Burton *et al.,* 2009) and in human babies (Lodha *et al.,* 2010).

No elevation of IL-6 was detected in sick lambs consistent with the results reported for equine leukocytes when the mRNA levels were not increased in the septic foals (Gold *et al.,* 2007). Kabaroff *et al.* (2006) showed that IL-6 serum concentration did not correlate at various *E. coli* LPS doses, when the results were compared with serum cortisol concentration or the febrile response. Recently, Tambuyzer *et al.* (2014) found that static absolute IL-6 concentrations in infected pigs were not different from the control group. In accordance with results obtained by other investigations we did not find any variation in the level of IL-10 in the serum from lambs. In septic foals, Burton *et al.* (2009) detected a wide range of IL-10 concentration, therefore, unlikely to provide a useful diagnostic indicator of sepsis when used alone. However, Pusterla *et al.* (2006) found that IL-10 was the only biological marker that could differ between sick non-septic and septic foals. In our study, sick lambs were non-septic and no significant difference in IL-10 concentration was identified. To confirm that IL-10 can be used as a biological marker in ovine diseases, an investigation with septic lambs is required.

The significant difference found in our study in IL-1β levels between sick and healthy lambs and the positive correlation between IL-6/IL-1β suggests that IL-1β can be used as a tool for the early detection of inflammatory diseases and evolution and assessment of the severity of the process in ovine pathology. In diarrheic lambs there was a high correlation between IL-6, IL-1β and serum protein profiles and this correlation was higher than that detected in the healthy lambs. This resulted as both cytokines are pro-inflammatory and are related to APP (Eckersall, 2008). Production of APP is controlled by cytokines IL-1, IL-6 and TNF-α released from the site of pathogenic damage. Sick lambs were infected by bacteria and parasites which caused diarrhea and these infections induced leakage of APP into the blood stream (Petersen *et al.,* 2004). However, measurement of any cytokines on a single occasion may not capture the actual peak levels reached during the disease process. Ideally, cytokine levels need to be measured serially to establish a correlation between rising levels of cytokines and severity of the diseases, such as suggested by Lodha *et al.* (2010) and Tambuyzer *et al.* (2014).

**Conclusions:** The cytokines follow a time-dependent evolution, being highest at days 1-4 after birth, then gradually decrease, except for IL-10. The present study indicated a strong correlation between IL-6 and IL-1β and serum protein fractions.

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**Author's contribution:** AF and DM conceived and designed the project, analyzed the final data. MRA and TC were the clinicians and took the blood sample, closely monitored the animal's health. DL and AL performed the laboratory analysis. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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