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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Evaluation of inflammatory biomarkers in goats naturally infected with *Babesia ovis*

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Abstract

This study was designed to evaluate the effects of *Babesia ovis* infection on concentrations of some essential acute phase proteins (APPs) including albumin, fibrinogen, serum amyloid A, haptoglobin, and ceruloplasmin as well as total, protein-binding, and lipid-binding sialic acids (TSA, PBSA, and LBSA) and two crucial cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Some hematological parameters also were evaluated. Furthermore, any probable correlation among the APPs, SAs, IFN-γ, and TNF-α was calculated. A total of 420 Marghoz and Raeini goats with the ages of 1–3 years old from the north and northwest of Iran were examined, and 17 goats confirmed to be infected with *B. ovis* by both routine microscopic examination of blood films and molecular assays. As the control, 17 healthy goats were included. The results revealed a significant decrease ($P < 0.05$) in erythrocyte count, hemoglobin level, and pack cell volume as well as a nonsignificant increase in white blood cell count in the diseased animals compared with the control. Additionally, all the APPs, SAs, and cytokines were remarkably higher in the infected animals than the uninfected ones, except for albumin, which was significantly lower. Moreover, a strong and positive correlation was detected among the parameters mentioned above, except for albumin, which was inversely correlated with the other parameters. In conclusion, *B. ovis* infection is associated with the induction of severe inflammatory reactions in goats, and both SA and APP are significantly involved in the pathophysiology of the disease.

Keywords Anemia · Acute phase protein · Cytokine · Sialic acid

Introduction

Ovine babesiosis is the most critical blood-borne parasitic disease of small ruminants in tropical and non-tropical regions, which is caused by *Babesia ovis*, *B. motasi*, and *B. crassa* in Iran (Dehkordi et al. 2010). Hemolytic anemia is the major consequence of babesiosis induced by mechanical damage to red blood cell (RBC) and increased membrane permeability due to oxidative stress, immune reactions, and erythrophagocytosis by activated macrophages (Esmaeilnejad et al. 2012b; Murase et al. 1996). Fever, anemia, hemoglobinuria, and jaundice are the main clinical signs, but asymptomatic infections are common (Chaudhuri et al. 2008).

Acute phase proteins (APPs) are a bunch of blood proteins which similar to the majority of other blood proteins are mainly synthesized in the liver (Eckersall 2000). Their concentrations can either increase (positive APPs) or decrease (negative APPs) following stimulation. As part of the early-defense or innate immune response, APPs production can be stimulated by various triggers such as trauma, infection, stress, neoplasia, and inflammation leading to a complex systemic reaction to reestablish homeostasis and promote healing(Cray et al. 2009). APPs have been

Highlights • Infection with *B. ovis* could increase the levels of fibrinogen, haptoglobin, serum amyloid A and ceruloplasmin, but suppressed albumin concentrations.
• Infection with *B. ovis* could also increase the levels of TNF-α, IFN-γ and sialic acids.
• All the acute phase proteins, sialic acids and cytokines were tightly correlated with each other.

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extensively studied in human medicine as a biomarker of inflammation, infection, and trauma, but in veterinary medicine, they have relatively been underutilized. However, significant progress has been made in the detection, measurement, and application of APPs as biomarkers in both companion and farm animal medicine over recent years (Eckersall and Bell 2010). For example, in dogs, large increases in C-reactive protein are associated with arthritis and lymphoma, and moderately elevated levels can be related to canine inflammatory bowel (Jergens et al. 2003). Moreover, in cattle, haptoglobin (Hp) is a useful tool in the diagnosis and prognosis of mastitis, enteritis, peritonitis, pneumonia, endocarditis, and endometritis (Murata et al. 2004).

The initiation of inflammatory processes by the innate immune system depends on cytokines and chemokines, which are secreted by activated cells including monocytes, macrophages, fibroblasts, endothelium, platelets, keratinocytes, and T cells (Striz et al. 2014). Alarmins and pro-inflammatory cytokines from the interleukin (IL)-1 and tumor necrosis factor (TNF) families initiate the cascade of events, including the induction of APPs release (Cray et al. 2009). It has been claimed that pro-inflammatory cytokines, particularly TNF-α, play an important role in the pathophysiology of animals' infection. According to Zygner et al. (2014), increased concentration of serum TNF-α is tightly associated with the development of hypotension and renal failure in dogs infected with *B. canis* (Zygner et al. 2014).

Sialic acid (SA), an acetylated derivative of neuraminic acid, can be detected in various tissues and body fluids of animals. The majority of SAs are in either protein-bounded (PBSA) or lipid-bounded (LBSA) forms, and only a minor portion is in the free forms. SA is also present at the end chain of many APPs (Crook 1993; Haq et al. 1993). The correlation between SA and APPs has previously been established in healthy human subjects and patients with myocardial infarction (Haq et al. 1993). However, in veterinary medicine, such determination has rarely been done.

Assessment of inflammatory biomarkers in domestic animals' piroplasmosis has aroused considerable research interest, because they may aid in early diagnosis or assessing response to treatment. As evidence, the levels of several cytokines and SA were recently measured in equine naturally infected with *Theileria equi* (Mostafavi et al. 2020). Additionally, SA contents and APPs have been evaluated in buffaloes, cattle, and sheep piroplasmosis (Esmaeilnejad and Froushani 2016; Esmaeilnejad et al. 2014a; Nazifi et al. 2009). However, such studies have not yet conducted in goats. Therefore, the current study was undertaken to assess APPs, SAs, TNF-α, and interferon-gamma (IFN-γ) in goats naturally infected with *B. ovis* and establish any correlation among the parameters mentioned above.

Material and methods

Animals and sampling

This study was conducted in rural areas of six different provinces, including Ardabil, East Azerbaijan, West Azerbaijan, Hamadan, Ilam, and Gilan, located in the north and northwest of Iran where piroplasmosis is prevalent during the seasons, early May to late September 2017–2019 (Fig. 1). A total of 420 Marghoz and Raeini goats (70 from each province) with the ages of 1–3 years old were examined, and 53 cases diagnosed to be infected with different *Babesia* and *Theileria* spp. The diagnosis was established on history, clinical signs, and light microscopic examination of blood films. For microscopic examination, thin blood smears from the ear vein of the animals were prepared and stained with Giemsa. At least, 100 oil-immersion fields were examined for the presence of *Babesia* spp., particularly the predominant existence of pyriform, single ring, and four daughter organisms (Longstaffe 1984). During sampling, the whole body of each animal was examined for the presence of tick infestation. The examined goats were raised under traditional husbandry practices (grazing on pastures during the day) without regular acaricide treatment. Blood specimens were collected by jugular venipuncture into sterile EDTA and plain tubes, then immediately placed on ice, and transferred to the laboratory. Serum samples were separated from plain tubes by centrifugation (1500×*g* for 10 min), then poured into Eppendorf tubes, and immediately stored at − 20 °C until analysis. All the specimens were analyzed maximally 72 h after the sampling. The animals had not been treated for the disease before sampling, and they were sampled once in the course of the disease. The common clinical signs of the infected animals were anorexia, fever (40–41.5 °C), different levels of anemia, and icterus. *B. ovis* infection was confirmed in the 17 cases (out of 53) using molecular assays and as a control group, 17 goats that had been clinically healthy over the last 6 months (according to history, clinical examination, blood smears, and biochemical tests) from the same farms, and matched sex, age, and breed were included in the study. The control animals were also negative for the presence of *B. ovis* in polymerase chain reaction (PCR) assay. All procedures in this study were carried out under the guidelines of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urmia University (IR-UU-AEC-219/DA3). Additionally, routine microbiological assessments such as direct microscopic blood examination with differential staining (Gram's and acid-fast staining methods), conventional pure culturing of whole blood (streaked onto blood agar medium), and routine biochemical tests were performed.

Fig. 1 The location of the provinces where the samples were collected

Molecular analysis

PCR was performed to confirm *B. ovis* infection. Briefly, after extraction of DNA, the following pair of primers, P1 (5'-CACAGGGAGGTAGTGACAAG-3′) and P2 (5'-AAGAATTTCACCTATGACAG-3′), were employed for amplification of *Babesia* spp. 18S ssu RNA (Schnittger et al. 2004). Semi-nested PCR was carried out for identification of *B. ovis* using the primers Bbo-F (5'-TGGGCAGGACCTTG GTTGTTCT-3′) and Bbo-R (5′-CCGCGTAGCGCCGG CTAAATA-3′) (Aktas et al. 2005). The PCR parameters for amplification were initial DNA denaturation over 5 min at 95 °C followed by 45 cycles of 45 s at 94 °C, 45 s at 63 °C, and 60 s at 72 °C, and terminated with a final extension step at 72 °C for 10 min. The obtained sequence was compared with available nucleotide sequences in GenBank using the Nucleotide Basic Local Alignment Search Tool program. The comparison revealed a 100% similarity between various sequences of *B. ovis* 18S rRNA gene deposited in GenBank (Accession numbers: MN611762.1, MG569902, etc.) indicating that the goats were infected with *B. ovis*. Under accession No: KY581551, the sequence of the PCR product was submitted to the GenBank database.

Assessment of hematological parameters and APPs

Hematological parameters including RBC and white blood cell (WBC) counts, hemoglobin (Hb) concentration, and packed cell volume (PCV) were determined by an automated hematology analyzer (MEK-6450 K-NIHON KOHDEN, Japan) and expressed in SI units.

Serum levels of APPs, including albumin, Hp, and serum amyloid A (SAA), were estimated based on the previously validated study (González et al. 2008) and commercially available assay kit. Briefly, Hp was assessed spectrophotometrically using a commercial kit (Tridelta Development Limited, Ireland) based on the peroxidase activity of the haptoglobin-hemoglobin complex. Albumin was measured based on bromocresol green method using a commercial kit (SPINREACT S.A., Gerona, Spain). SAA levels were evaluated using a commercial ELISA kit (Tridelta Development Limited, Bray, Ireland). Heating precipitation method was employed for the assessment of fibrinogen levels in plasma samples (Millar et al. 1971). Ceruloplasmin (Cp) was measured according to the method described by Sunderman Jr. and Nomoto (1970), which is based on the measurement of p-phenylenediamine oxidase activity (Sunderman Jr. and Nomoto 1970).

Assessment of SA and cytokines

Serum total SA (TSA) levels were measured based on the thiobarbituric acid method described previously (Warren 1959), and the standard curve was created using a standard sample of N-acetyl neuraminic acid. The method developed by Katopodis and others (1982) was employed for the

estimation of LBSA. The above-mentioned standard curve was also used for assessing LBSA (Voigtmann et al. 1989). PBSA was measured by subtracting serum TSA from LBSA. The concentrations of TNF-α and IFN-γ were measured using double-sandwich ELISA (Cat No. MBS263127) and quantitative sandwich ELISA (Cat No. MBS701155) kits, respectively, based on the instructions provided by the manufacturer (MyBioSource, San Diego, USA) and expressed as pg/mL (goat TNF-α ELISA kit, sensitivity: 0.05 ng/mL and goat IFN-γ, sensitivity: typically less than 1.95 pg/mL).

Statistical analysis

The packaged SPSS program for Windows (version 22. 2013, Chicago, IL, USA) was employed for statistical analysis. Data were expressed as mean and standard deviation (means ± SD). The data normality was assessed using the Shapiro-Wilk test and considering the normal distribution of the data, Student's *t* test was employed to estimate the difference between the infected and uninfected animals. Pearson's correlation (r) was carried out on the paired data obtained by individual infected cases. $P < 0.05$ was considered statistically significant.

Results

Both the routine microscopic examination and PCR assay confirmed *B. ovis* infection in the 17 goats (Fig. 2). The parasitemia was determined to be in the range of 0.01 to 1%. Additionally, no sample showed microscopic evidence of other potential causes of anemia. Also, no growth was seen on blood agar medium after the culture of samples and the results of routine biochemical tests were well within the normal range.

The values of hematological parameters in the healthy (control) and infected goats are depicted in Table 1. As presented, RBC count, PCV, and Hb levels were significantly declined in the diseased animals compared with those of healthy ones; however, WBC count was nonsignificantly higher in these animals. Table 2 represents the levels of various APPs measured in this study. As can be seen, all the APPs were significantly elevated in the *B. ovis* infected goats as compared with those of healthy animals except for albumin, which was reduced. Particularly, SAA and Hp were roughly seven- and eightfold higher in the infected animals than the uninfected goats, respectively. As shown in Table 3, the amounts of different SAs, and cytokines, were significantly raised in the diseased goats as compared with the control. Pearson correlation test revealed a strong and positive correlation among APPs, SAs, and the cytokines, except for albumin, which was inversely correlated with the other parameters (Table 4).

Discussion

The available evidence suggests that both systemic inflammation and oxidative stress are involved in the pathogenesis of equine, ovine, bovine, and canine piroplasmosis (Chaudhuri et al. 2008; Esmaeilnejad et al. 2014a, b; Mostafavi et al. 2020; Zygner et al. 2014). The status of lipid peroxidation and activity of antioxidant enzymes were previously measured in goats naturally infected with *B. ovis* (Esmaeilnejad et al. 2012b). However, the inflammatory biomarkers have not yet been adequately addressed in caprine babesiosis. Therefore, we aimed at this shortcoming and evaluated several inflammatory biomarkers in goats naturally infected with *B. ovis*.

The infection could remarkably decrease RBC count, Hb concentration, and PCV as compared with the uninfected animals. This finding indicates anemia and is consistent with previous reports (Esmaeilnejad et al. 2012a, b). The observed anemia can be related to immunological reactions in which autoantibodies are produced against the components of erythrocyte membranes or toxic hemolytic factors produced by the parasite or mechanical damage due to propagation of trophozoite in RBC and erythrophagocytosis (Esmaeilnejad et al. 2012a). Increased oxidative stress in RBCs during the infection can undermine the integrity of the RBC membrane and

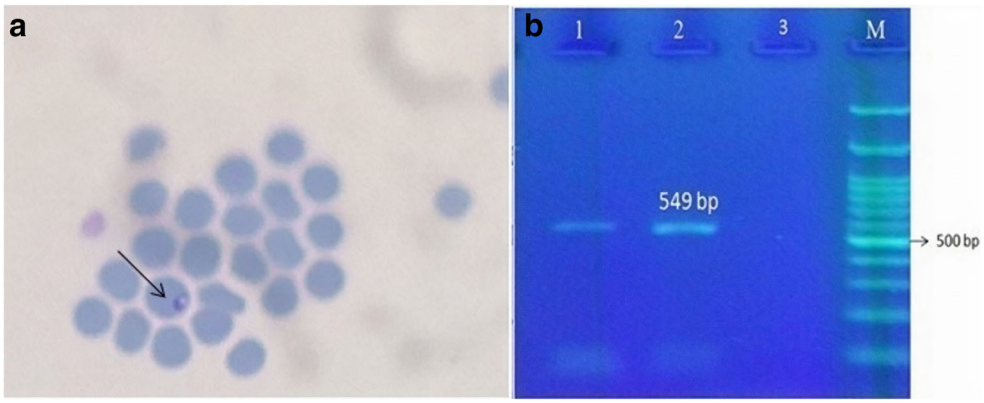

Fig. 2 **a** *Babesia* spp. inside goat RBC (Giemsa, × 1000). **b** Simple PCR for detection of *B. ovis*. Lane M: molecular marker (100 bp ladder); Lane 1: *B. ovis*-infected blood. Lane 2: *B. ovis* positive control; Lane 3: negative control

Table 1 Hematological parameters of healthy goats and those of naturally infected with *B. ovis*

Goats	RBC (10^{12}/L)	WBC (10^{9}/L)	PCV (L/L)	Hb (g/L)
Healthy ($n = 17$)	6.41 ± 0.57	7.62 ± 0.61	0.28 ± 0.03	93.54 ± 6.12
Infected ($n = 17$)	4.29 ± 0.33[a]	8.32 ± 0.42	0.20 ± 0.01[a]	68.49 ± 5.73[a]

[a] Denotes significant differences ($P < 0.05$)

make it vulnerable to lysis leading to hemolytic anemia (Esmaeilnejad et al. 2012b; Otsuka et al. 2001). The leukogram of the diseased animals showed a nonsignificant increase in WBC count compared with those of healthy goats. In agreement, a parasitemia-dependent increase in WBC count has been reported in small ruminants naturally infected with *B. ovis* (Esmaeilnejad et al. 2012a). The leukocytosis can be due to the maturation of neutrophils and lymphocytes (Furlanello et al. 2005).

Measurements of APPs can be served as a potential diagnostic tool in veterinary medicine, but one should take care while assessing APPs, because the acute phase response varies in different species and different pathological processes (Murata et al. 2004). Although APPs are nonspecific indicators of biological reactants, they can be employed for assessing nutritional deficits and reactive processes (Gruys et al. 2005). For instance, APPs have been measured in milk and serum of dairy cows with clinical mastitis and it was noticed that the diagnostic value of Hp in differentiating between the healthy and diseased animals gave high sensitivities and specificities ranging from 82 to 100% (Eckersall et al. 2001). In this study, we measured significantly elevated concentrations of fibrinogen, Hp, SAA, and Cp in the infected goats compared with the control group suggesting induction of severe inflammatory reactions. In support of our findings, raised levels of APPs were measured in goats with naturally acquired *Staphylococcus aureus* mastitis and those of aborting and non-aborting goats infected with border disease virus (Balikci et al. 2013; Simplício et al. 2017). Notably, we found that SAA and Hp levels were seven- and eightfold higher in the diseased animals, respectively. SAA proteins are a family of apolipoproteins associated with high-density lipoprotein in plasma. Its production is stimulated by pro-inflammatory cytokines, such as IL-6, IL-1, TNF, IFN-γ, and transforming growth factor-β (Targońska-Stępniak and Majdan 2014). Hp is an α2-glycoprotein which binds to free Hb to form a complex to prevent iron excretion through the kidneys (Weinberg and D'Angio 2011). It is depleted when the daily Hb turnover increases roughly twice the normal rate, regardless of type of the hemolysis (extravascular or intravascular) (Lewis and Roper 2006). We measured increased levels of Hp in the infected animals despite the hemolytic anemia. In agreement, increased Hp levels have been reported in other hemolytic conditions such as bovine theileriosis and canine dirofilariasis (El-Deeb and Iacob 2012; Mendez et al. 2014). It is unclear how Hp is increased during the above-mentioned diseases; however, in an effort to elucidate the association between the Hp and severity of human malaria, Mendonça et al. (2012) revealed that during an acute malarial attack, the levels of Hp is extensively increased to cope with circulating free Hb (Mendonça et al. 2012).

Positive APPs can be further classified as major, moderate, or minor, depending on the magnitude of elevation during the acute phase response. It has been claimed that both SAA and Hp are major APPs in goat and fibrinogen is a moderate one (Cray et al. 2009). Albumin as a negative APP was measured to be decreased in this study, which was consistent with the previous report (Esmaeilnejad et al. 2012a). The reduction can be attributed to selective loss of albumin following renal or gastrointestinal changes or a decrease in hepatic synthesis (Cray et al. 2009). The increased level of TNF-α and IFN-γ as observed in this study may indicate that the monocytes and macrophages were stimulated to secret pro-inflammatory cytokines in response to a stressful situation (the parasitic infection) (Peraçoli et al. 2003). The obtained data was comparable with those goats infected with *Coxiella burnetii* and cattle infected with *Anaplasma marginale* (El-Deeb et al. 2019; Nazifi et al. 2012b).

In the present study, the serum SA concentrations were higher in the infected goats than those of control animals ($P < 0.05$). It is not clear how the infection with *B. ovis* can increase SA contents; however, the studies conducted on

Table 2 Acute phase proteins of healthy goats and those of naturally infected with *B. ovis*

Goats	Albumin (g/L)	Fibrinogen (g/L)	SAA (mg/L)	Hp (g/L)	Cp (mg/dl)
Healthy ($n = 17$)	30.13 ± 2.74	9.12 ± 0.97	86.39 ± 7.08	2.53 ± 0.11	8.57 ± 0.64
Infected ($n = 17$)	22.41 ± 2.29[a]	25.94 ± 3.61[a]	679.28 ± 11.68[a]	16.29 ± 1.89[a]	70.05 ± 9.93[a]

[a] Denotes significant differences ($P < 0.05$)

Hp haptoglobin, *SAA* serum amyloid A, *Cp* ceruloplasmin

Table 3 Sialic acids and cytokines of healthy goats and those of naturally infected with *B. ovis*

Goats	TSA	PBSA	LBSA	IFN-γ (pg/mL)	TNF-α (pg/mL)
Healthy (n = 17)	2.06 ± 0.32	1.03 ± 0.18	1.04 ± 0.21	21.52 ± 2.17	9.33 ± 1.56
Infected (n = 17)	3.74 ± 0.54[a]	1.89 ± 0.33[a]	1.86 ± 0.29[a]	40.09 ± 2.88[a]	16.49 ± 2.79[a]

TSA total sialic acid, *PBSA* protein-binding sialic acid, *LBSA* lipid-binding sialic acid, *IFN-γ* interferon gamma, *TNF-α* tumor necrosis factor alpha

[a] Denotes significant differences ($P < 0.05$)

ovine babesiosis suggest that the increased level of serum SAs may alter receptor-ligand interactions, which are known to play a central role in the inflammatory response (Esmaeilnejad et al. 2014a). As mentioned above, stimulation of local cytokine secretion occur following tissue injuries, which induces an acute-phase response, including the release of APPs that bind to SA in the liver and thereby are transferred to the general circulation, again leading to increased SA concentration (Crook et al. 2001). We observed a strong and positive correlation among APPs (except for albumin), SAs, and the cytokines. Such assessment has rarely been done in veterinary medicine, and our finding is consistent with the study carried out by Nazifi and colleagues (Nazifi et al. 2012a, b) who detected the same correlation in cattle infected with foot-and-mouth disease (Nazifi et al. 2012a).

Our data clearly shows that *B. ovis* infection induces systemic inflammation in goats, evidenced by a remarkable increase in the cytokines, SA, and APP (except for albumin) contents in the infected animals. This is an expected finding and is parallel to observations of other researchers who reported systemic inflammation following babesiosis. To be specific, Goddard et al. (2016) demonstrated that *B. rossi* infection causes a severe inflammatory response in dogs and various cytokines undergo dramatic changes. According to their results, some of the cytokines increase, while the others decrease during the infection (Goddard et al. 2016). Additionally, Leisewitz et al. (2019) have claimed that generally, the more complicated the disease, the more pro-inflammatory the cytokine milieu and TNF-α is significantly higher in the dogs that died due to *B. rossi* infection, compared with the dogs that survived (Leisewitz et al. 2019). Moreover, Schetters et al. (2009) revealed that systemic inflammatory responses in dogs experimentally infected with *B. canis* were associated with elevation of APPs levels, especially fibrinogenaemia (Schetters et al. 2009).

The small sample size was one of the inevitable limitations of this study. It should be noted that the prevalence of *B. ovis* in goats is much lower than sheep. As evidence, in our previous study conducted on the northwest of Iran (the same region), we could detect only 15 positive cases out of 122 animals using PCR (Esmaeilnejad et al. 2015). Similarly, Aktas et al. (2006) identified only 1 positive case out of 100 goats in our neighbor country, Turkey (Aktas et al. 2006). Considering that the prevalence of *B. ovis* infection is extremely rare in industrial farms (almost zero), the current study was conducted in rural areas where the goats were raised under traditional system (grazing in pastures from sunrise to sunset). Several factors such as environmental stressors and nutrition would influence the levels of cytokines, APPs, and SAs. More importantly, it is not clear whether the animals were infected for

Table 4 Correlation among acute phase proteins, sialic acids and cytokines in goats naturally infected with *B. ovis* (Pearson correlation)

parameter	Albumin	Fibrinogen	SAA	HAP	CER	TSA	PBSA	LBSA	IFN-ϒ	TNF-α
Albumin	1	−0.882*	−0.913*	−0.911*	−0.911*	−0.849*	−0.833*	−0.829*	−0.893*	−0.880*
Fibrinogen		1	0.991*	0.996*	0.995*	0.969*	0.963*	0.936*	0.997*	0.995*
SAA			1	0.995*	0.998*	0.960*	0.960*	0.922*	0.995*	0.989*
HAP				1	0.998*	0.964*	0.956*	0.932*	0.997*	0.991*
CER					1	0.964*	0.960*	0.982*	0.996*	0.992*
TSA						1	0.978*	0.982*	0.962*	0.961*
PBSA							1	0.923*	0.959*	0.956*
LBSA								1	0.925*	0.925*
IFN-ϒ									1	0.992*
TNF-α										1

Hp haptoglobin, *SAA* serum amyloid A, *Cp* ceruloplasmin, *TSA* total sialic acid, *PBSA* protein-binding sialic acid, *LBSA* lipid-binding sialic acid, *IFN-γ* interferon gamma, *TNF-α* tumor necrosis factor alpha

*Correlation is significant at the 0.01 level (2-tailed)

the first time or not, as there would probably be a difference between the first-time infection and reinfection. We performed some additional tests (routine microbiological and biochemical assays) to ensure that there was no coinfection with bacteria or any background health problem; therefore, every change in the levels of cytokines, APPs, and SAs could be related to *B. ovis* infection. However, we could not control all the variables and confounding factors, which should be considered in future studies.

To summarize, the results demonstrated that the inflammatory mediators (TNF-α, INF-γ, and SAs) and APP (SAA, Hp, Ce, and fibrinogen) concentrations were raised in *B. ovis*-infected goats in response to the parasitic infection. Additionally, the parameters were tightly correlated with each other. However, albumin as a negative APP was reduced in the infected animals and had an inverse correlation with the other indices. SAs and APPs are significantly involved in the pathophysiology of the disease, but this study cannot explain their exact role. Furthermore, it is not clear by which mechanisms SAs were increased. Such evaluations should be conducted in future studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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