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Short Communication

Haematological Changes and Hyperbilirubinaemia in Sheep Naturally Infected with *Babesia ovis* in an Enzootic Focus

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ABSTRACT

Malignant ovine babesiosis is a seasonal tick-borne disease associated with anaemia and potentially high mortality. The aim of this study was to characterise haematological changes and total bilirubin concentrations in naturally infected sheep originating from an enzootic focus of Rudo in Bosnia and Herzegovina. Twenty PCR-confirmed sheep showing clinical signs compatible with malignant ovine babesiosis and ten clinically healthy, PCR-negative control sheep were examined. Blood samples were collected from the jugular vein into EDTA tubes, and haematological parameters were determined using an automated analyser, while total bilirubin was measured by reflectometry. Parasitaemia was assessed microscopically on Giemsa-stained blood smears using a semi-quantitative scale. All infected animals were assigned to the lowest parasitaemia category (<1%). The infected sheep had significantly lower erythrocyte count, haemoglobin concentration and haematocrit than controls, while the mean corpuscular volume did not differ significantly between the groups. The most prominent finding was severe thrombocytopenia, with a mean platelet count of 91.95 × 10 ^{9}/L compared with 426.40 × 10 ^{9}/L in controls. The total bilirubin concentration was also significantly higher in the infected sheep. The marked thrombocytopenia observed despite low microscopically detected parasitaemia suggests that mechanisms other than parasite burden alone may contribute to platelet depletion, although the underlying mechanism cannot be established from the present data. The findings provide laboratory data from naturally occurring malignant ovine babesiosis in an enzootic focus in Bosnia and Herzegovina and highlight severe thrombocytopenia and hyperbilirubinaemia as prominent findings.

Key words:
sheep; babesiosis; thrombocytopenia; bilirubin; haematology; tick-borne diseases

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INTRODUCTION

Babesia ovis causes severe disease in sheep, better known as malignant ovine babesiosis, characterised by depression, fever, anaemia, haemoglobinuria and icterus (Bilgic et al., 2020; Stevanović et al., 2022). It is a typical seasonal, vector-borne disease with high mortality, and the principal vector for *B. ovis* in Bosnia and Herzegovina is *Rhipicephalus bursa* (Stevanović, 2023). Although the disease has been known for more than 130 years, detailed clinical studies remain scarce. Haematological and biochemical changes in sheep naturally infected with *B. ovis* have been studied in Israel (Yeruham et al., 1998), Turkey (Sevinc et al., 2013), Iran (Esmaeilnejad et al., 2012) and Pakistan (Sajid et al., 2023), but no comparable studies exist in the Balkans.
Babesia ovis shows variation in the virulence of individual isolates originating from different geographical and epizootiological regions; consequently, the changes in blood count and biochemical parameters are not necessarily

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highly specific. Isolates from Bulgaria have been reported as highly pathogenic, although the cause of these differences in virulence has never been clarified (Kiurtov et al., 1981). Assessing virulence and its influence on clinical severity under natural infection is difficult and requires prolonged epizootiological, immunological and clinical investigation, while determination under experimental conditions is limited by the small number of experimental animals. Differences in the severity and course of malignant ovine babesiosis have been established between Rudo, where an isolated enzootic focus has formed, and the Herzegovina region (Stevanović, 2023). In this focus, the disease typically takes a fulminant course with high lethality despite treatment with imidocarb dipropionate, and shows no clear age or breed predisposition (Stevanović, 2023). Mixed infection with *Babesia ovis* and *Anaplasma ovis* has also been demonstrated in this area, although it did not affect the clinical outcome (Stevanović et al., 2024).

The aim of this study was to investigate the haematological alterations and changes in total bilirubin concentration in sheep infected with *B. ovis* originating from an isolated enzootic focus, and to compare them with a clinically healthy, PCR-negative control group.

MATERIAL AND METHODS

This study was based on routine field diagnostics carried out during the regular work of the epizootiology service of the Public Institution Veterinary Institute of the Republic of Srpska "Dr Vaso Butozan", Banja Luka. As the investigation did not constitute a purpose-designed animal experiment but rather routine diagnostic sampling of naturally infected clinical cases, formal ethics-committee approval was not required.

The study included 20 sheep in which *B. ovis* infection was confirmed by a conventional PCR protocol (Aktaş et al., 2005). Briefly, a 549 bp fragment of the 18S rRNA gene was amplified with the *B. ovis*–specific primers and cycling conditions described by Aktaş et al. (2005), in a total reaction volume of 25 µl of HiChrom PCR Master Mix (HiMedia / HiGenoMB, India) on a Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany). Positive and negative controls were included in each run. DNA from *Babesia ovis* (GenBank accession no. MZ853105) was used as the positive control. Products were visualised by electrophoresis in a 1.5 % agarose gel stained with Midori Green (Nippon Genetics Europe, Düren, Germany).

The examined animals displayed clinical signs of malignant ovine babesiosis: depression, anorexia and fever (≥40 °C). All sheep originated from the enzootic focus in Rudo; two sheep were sampled in Rogatica (the village of Žepa), where the cases had been linked to illegal animal transport from Rudo (Stevanović, 2023). Of the 20 infected sheep, 13 were female and 7 were male, aged from 4 months to 4 years. The breed structure comprised Pramenka (18 sheep) and Merino (2 sheep).

The control group consisted of 10 clinically healthy sheep from two flocks kept under typical extensive, pasture-based management. All control animals were female and older than two years, were negative for *B. ovis* by PCR, and were free of tick infestation at the time of sampling. Additional laboratory assessment of the health status of the control group (parasitological examination and diagnostics for other infectious diseases) was not performed. The control group was not individually formed to the infected animals for age, sex, breed, reproductive status or flock of origin because the sampling was based on field diagnostic of disease. The study was not prospectively designed as case-control study.

Blood samples were collected from the jugular vein into EDTA tubes and used for PCR, microscopy, and haematological and biochemical analyses. The parasitaemia level was determined on Giemsa-stained smears according to Aktaş et al. (2005), with approximately 1,000 erythrocytes examined per smear, and graded according to Esmaeilnejad et al. (2012) as <1%, 1%, 2% and 3%. The lowest category represents a broad range of low parasite loads, so the microscopic assessment was considered semi-quantitative rather than an exact measurement. Platelet counts were verified by light-microscopic examination of Giemsa-stained smears under oil immersion to exclude pseudothrombocytopenia, and the smears were additionally screened for platelet aggregates.

Haematological analysis was performed using an automated analyser (scil Vet abc, Antech Diagnostics, Germany). Total serum bilirubin concentration was measured using the reflectometric test-strip system of the Reflotron Plus automated analyser (Roche Diagnostics GmbH, Germany).

Data were processed in Microsoft Excel (Microsoft Corp., USA) and GraphPad Prism (GraphPad Software, USA). For each parameter, descriptive statistics were calculated, comprising the mean, standard deviation (SD), minimum–maximum range and coefficient of variation (CV = SD / mean × 100). The normality of each variable was assessed using the Shapiro–Wilk test. Differences between the infected and control groups were evaluated using the independent-samples Student's *t*-test for normally distributed variables and the Mann–Whitney *U* test for variables that were not normally distributed. $p < 0.05$ was considered statistically significant. Ovine reference intervals were taken from Kaneko et al. (2008) and Jain (1993).

RESULTS AND DISCUSSION

All 20 sheep with PCR-confirmed *B. ovis* infection had a parasitaemia level below 1%, and no animal exceeded the lowest grade of the scale of Esmaeilnejad et al. (2012). Microscopic examination of the blood smears revealed various morphological forms of *B. ovis* merozoites, with single spherical forms being the most common. The typical paired pear-shaped (piriform) merozoites were observed rarely (Figure 1). The principal haematological and biochemical findings, together with the comparison with the control group, are presented in Table 1.

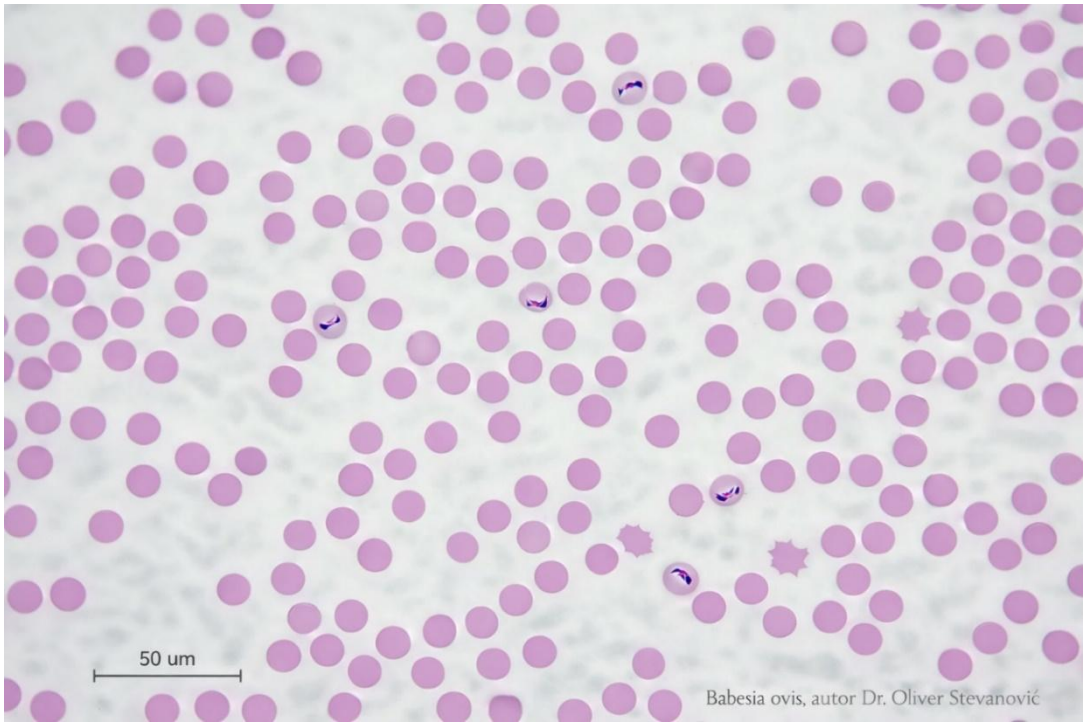


Figure 1. Merozoites of *Babesia ovis* in a Giemsa-stained blood smear - Rudo isolate (x1000)

Table 1. Principal haematological and biochemical findings in *Babesia ovis*-infected sheep and the control group.

Parameter	Unit	Infected		Control		p value	Ovine reference[1]
		Mean ± SD	CV %	Mean ± SD	CV %		
WBC (White blood cells)	$\times 10^9$/L	7.89 ± 3.09	39.1	10.27 ± 3.12	30.4	0.063	4.0–12.0
RBC (Red blood cells)	$\times 10^{12}$/L	**7.66 ± 1.40**	18.3	9.60 ± 0.82	8.5	**<0.001**	9.0–15.0
HGB (Hemoglobin)	g/dL	**8.06 ± 1.42**	17.6	9.19 ± 1.02	11.1	**0.017**	9.0–15.0
HCT (Hematocrit)	%	**23.78 ± 4.28**	18.0	30.28 ± 2.77	9.2	**<0.001**	27–45
PLT (Platelets)	$\times 10^9$/L	**91.95 ± 50.05**	54.4	426.40 ± 236.20	55.4	**0.001**	250–750
MCV (Mean corpuscular volumen))	fL	31.15 ± 1.73	5.5	31.60 ± 0.70	2.2	0.418	28–40
MCH (Mean corpuscular	pg	10.53 ± 0.70	6.7	9.54 ± 0.39	4.1	**<0.001**	8–12

Parameter	Unit	Infected		Control		p value	Ovine reference[1]
		Mean ± SD	CV %	Mean ± SD	CV %		
hemoglobin conentration)							
MCHC (Mean corpuscular hemoglobin conentration)	g/dL	34.11 ± 2.68	7.9	30.33 ± 1.53	5.1	**<0.001**	31–34
Total bilirubin[2]	mg/dL	**3.14 ± 1.68**	53.4	0.96 ± 0.30	31.2	**0.001**	0.1–0.5

Legend: [1] Ovine reference intervals (Kaneko et al., 2008; Jain, 1993). [2] Total bilirubin measured in 11 infected and 7 control animals; all other parameters n = 20 (infected) and n = 10 (control). CV = (SD / mean) × 100. Both groups were processed on the same analyser.

The infected group showed a marked anaemia. Erythrocyte count (7.66 ± 1.40 $\times 10^{12}$/L), haemoglobin concentration (8.06 ± 1.42 g/dL) and haematocrit (23.78 ± 4.28 %) were all significantly lower than in the control group ($p < 0.001$ for RBC and HCT; $p = 0.017$ for HGB). The mean haematocrit and haemoglobin values in the infected sheep fell below the ovine reference intervals, whereas those of the control group remained within the reference range. The mean corpuscular volume did not differ significantly between groups ($p = 0.418$), while mean corpuscular haemoglobin (10.53 ± 0.70 pg) and mean corpuscular haemoglobin concentration (34.11 ± 2.68 g/dL) were significantly higher in the infected group (both $p < 0.001$), indicating a normocytic, normochromic character of the anaemia in contrast to the lower corpuscular indices in the control animals.

The most pronounced finding was a severe thrombocytopenia (mean 91.95 ± 50.05 $\times 10^9$/L), markedly below both the ovine reference interval and the control group (426.40 ± 236.20 $\times 10^9$/L; $p = 0.001$). The smear evaluation confirmed consistently scarce platelets without aggregates, excluding pseudothrombocytopenia. The platelet count showed the highest variability among the haematological parameters (CV = 54.4 %).

The total leukocyte count did not differ significantly between the infected (7.89 ± 3.09 $\times 10^9$/L) and control groups ($p = 0.063$) and remained within the reference range, although it exhibited a high coefficient of variation (CV = 39.1 %), with individual values ranging from leukopenia to leukocytosis.

Total serum bilirubin concentration was significantly elevated in the infected group (3.14 ± 1.68 mg/dL) compared with the control group (0.96 ± 0.30 mg/dL; $p = 0.001$), exceeding the ovine reference interval several-fold and consistent with the haemolytic nature of the disease.

To the best of the authors' knowledge, this is the first study to characterise the haematological and biochemical changes of naturally occurring malignant ovine babesiosis from an enzootic focus in the Balkan region. The infected sheep showed a consistent pattern of anaemia, severe thrombocytopenia and hyperbilirubinaemia, in agreement with the clinical presentation of ovine babesiosis described in the literature (Esmaeilnejad et al., 2012; Sevinc et al., 2013; Sajid et al., 2023).

The anaemia observed was normocytic and normochromic, as indicated by the mean corpuscular volume remaining within the reference range while the erythrocyte count, haemoglobin and haematocrit were significantly reduced. This is consistent with Sevinc et al. (2013), who associated natural *B. ovis* infection in sheep with a normocytic–normochromic anaemia. The significantly higher MCH and MCHC in the infected group compared with the controls further support a normochromic, haemolytic process, distinct from the lower corpuscular indices seen in the control animals. The presence of severe clinical disease despite low microscopically detected peripheral parasitaemia may have several explanations. These include the limited sensitivity of microscopic examination at low parasite densities, uneven distribution or sequestration of infected erythrocytes, the timing of blood collection in relation to disease progression, differences in parasite virulence, and host-related factors.

The most prominent laboratory finding was severe thrombocytopenia (mean 91.95 × 10^9/L), which was markedly lower than both the ovine reference interval and the control group. All infected animals were assigned to the lowest microscopic parasitaemia category (<1%). The marked thrombocytopenia in this setting suggests that parasite burden alone may not fully explain the observed reduction in platelet count. Immune-mediated platelet destruction, increased platelet consumption, splenic sequestration, endothelial activation, coagulation abnormalities and systemic inflammatory responses represent possible mechanisms that require further investigation. Thrombocytopenia is recognised as a significant haematological manifestation of babesiosis in both dogs and sheep, and has been consistently documented in *B. ovis* infection (Sevinc et al., 2013; Sajid et al., 2023). The dissociation between the

severity of thrombocytopenia and the parasitaemic load suggests that platelet depletion is driven not primarily by direct parasitic destruction of host cells, but rather by immune-mediated destruction and consumption. Notably, the present results show a more pronounced thrombocytopenia (below 100 $\times 10^9$/L) than reported from Türkiye and Pakistan (Sevinc et al., 2013; Sajid et al., 2023). This finding supports previous clinical observations from the same enzootic focus, which described a fulminant disease course with high lethality (Stevanović, 2023). The high inter-individual variability of the platelet count (CV = 54.4 %) is in line with the variable platelet response reported between studies and geographic regions; the platelet indices reported by Sajid et al. (2023) were in contrast to those of Sevinc et al. (2013).

The total leukocyte count did not differ significantly between groups and remained within the reference range. However, total leukocyte count alone provides limited information about the inflammatory response, and differential leukocyte counts were not available in the present study. The leukocyte response in *B. ovis* infection is itself inconsistent in the literature, ranging from leukopenia (Sevinc et al., 2013) to leukocytosis (Esmaeilnejad et al., 2012). The high coefficient of variation in our study is consistent with this variability, and the absence of a significant difference may also reflect the limited size of the control group.

Total serum bilirubin concentration was significantly higher in the infected group than in the controls and exceeded the ovine reference interval several-fold. Similar observations were reported in literature (Sevinc et al., 2013; Sajid et al., 2023). The finding is compatible with haemolysis, which is a recognised feature of babesiosis, but the precise mechanism of the bilirubin increase cannot be established because only total bilirubin was measured. Despite these high concentrations, clinical examination did not reveal icterus of the visible mucous membranes or skin. This may be explained by the fulminant course of the disease, in which active deposition of bilirubin in tissues had not yet occurred, as such signs become apparent only after several days (Stevanović, 2023).

An important limitation of the present study is the lack of individual comparison between infected and control animals. Age, sex, breed, nutritional status, physiological status and flock-level management may influence haematological and biochemical variables and may therefore act as potential confounding factors in this study. The health status of the control animals was assessed only clinically and by PCR testing for *B. ovis*. Therefore, subclinical infections or parasitic conditions affecting haematological or biochemical parameters cannot be completely excluded.

CONCLUSION

Naturally occurring malignant ovine babesiosis in the examined sheep from the Rudo enzootic focus was characterised by anaemia, marked hyperbilirubinaemia and severe thrombocytopenia despite low microscopically detected parasitaemia. These findings suggest that parasite burden alone may not fully explain the observed platelet depletion. However, the underlying mechanisms remain unclear. Given the small sample size and the limitations of the control group, further studies involving larger, better-matched populations are needed.

Authors' Contribution: Conceptualization, writing and editing of final version of manuscript was done by Oliver Stevanović.

Ethical Statement: The study was based on routine field diagnostics performed during the regular work of the epizootiology service of the Public Institution Veterinary Institute of the Republic of Srpska "Dr Vaso Butozan", Banja Luka. As it did not constitute a purpose-designed animal experiment but routine diagnostic sampling of naturally infected clinical cases, formal ethics-committee approval was not required.

Data availability statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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

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