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First molecular evidence of hemotropic *mycoplasmas* in goats from Bosnia and Herzegovina

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ABSTRACT

Background: Hemoplasmas represent the type of bacteria that infect red blood cells, potentially leading to various health impacts, including changes in blood parameters. The close interaction between hemoplasma and red blood cells results in cell damage through immune-related and other unspecified mechanisms. Even with a strong immune response and antibiotic treatment, affected animals are likely to remain chronic carriers once clinical symptoms have subsided. These microorganisms were previously documented in sheep and other small ruminants worldwide.

Aim: Since there is a lack of research on the link between *Mycoplasma* infection and blood parameters, our aim was to investigate how *Mycoplasma* infection affects these blood parameters. In addition, the study conducted in Bosnia and Herzegovina represents the first documented research of hemoplasma infection in goats within this region.

Methods: In this research, 20 Alpine goats were sampled to investigate the presence of hemoplasma using polymerase chain reaction (PCR) analysis. Sequences of the 16S rRNA gene fragments were identified subsequently. The effect of *Mycoplasma ovis* (*M. ovis*) infection was observed on the following hematological parameters: Red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean cell volume (MCV), mean cell hemoglobin, mean cell hemoglobin concentration, Reticulocyte count, and white blood cell (WBC). Effect on white blood cell differentiation, absolute white blood cell counts, platelet count, and mean platelet volume were also investigated.

Results: PCR analysis confirmed the presence of *Mycoplasma spp*. in 7 out of the 20 blood samples. Sequencing of the 16S rRNA gene fragments revealed that all positive samples were identified as *M. ovis*. The research findings highlighted potential effects on blood parameters in infected goats. Goats infected with *M. ovis* exhibited higher mean levels of HGB and HCT compared to uninfected goats. However, there were no statistically significant differences in RBC counts between infected and uninfected groups. The study also noted significantly higher WBC counts in goats without *M. ovis* infection.

Conclusion: 35% of animals tested positive for *M. ovis*. Our study's findings showed notable differences in hematological parameters between goats infected with *M. ovis* and those that were not infected.

Keywords: Bosnia and Herzegovina, Goats, Hematological parameters, Hemoplasma infection, *Mycoplasma ovis*.

Introduction

Goats play a crucial role in mixed farming systems due to their adaptability, resistance to pathogens, and efficient feed conversion. Despite the favorable conditions for raising and breeding goats in every part of Bosnia and Herzegovina, the current goat population in the country is approximately 73,000, according to data from the Statistical Agency of Bosnia and Herzegovina (Livnjak and Hadžimusić, 2022). The herds in Bosnia and Herzegovina consist mainly of Alpine goats that are primarily used for milk production. Available data suggests that herds usually range between 50 and 200 goats, although there is a rare instance of a single herd comprising over 700 animals.

The most commonly found hemotropic *mycoplasma*s (hemoplasmas) in goats and various other small ruminants are *Mycoplasma ovis* (*M. ovis*) and *Candidatus Mycoplasma* haemovis. These non-cultivatable small bacteria are pleomorphic, wall-less, and reside in the erythrocytes infecting various vertebrate hosts. Previously, they were classified under the *Anaplasmataceae* family as *Haemobartonella* and *Eperythrozoon*, but they have since been reclassified as *Mycoplasma* due to their phylogenetic placement based on the 16S rRNA gene sequences (Galon *et al.*, 2019).

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Hemoplasmosis is typically transmitted naturally via arthropods including ticks, mosquitoes, horseflies, and certain other biting flies. However, hemoplasmas can also be transmitted mechanically. Iatrogenic transmission may be facilitated through fomites, i.e., hypodermic needles and equipment used for tail-docking, castrating, or disbudding animals (Shi *et al.*, 2023). The severity of hemoplasma infection varies depending on the susceptibility of the host and the pathogenicity of the species.

Mycoplasma ovis and *Candidatus Mycoplasma haemovis* are recognized as causes of hemolytic anemia and decreased exercise tolerance in sheep suffering from acute infections (Byamukama *et al.*, 2020). However, clinical signs are rarely seen in goats (Stuen, 2016). Infected goats, on the other hand, often show mild clinical signs and may become persistent carriers due to low levels of bacteremia (Machado *et al.*, 2017). Hemoplasma infections have a considerable effect on small ruminant production, primarily due to the mortality and productivity losses observed in chronically infected animals. (Shi *et al.*, 2019). To the best of the authors' knowledge, there are no documented cases of hemotropic *Mycoplasma* in goats from Bosnia and Herzegovina. Additionally, information on the effects of these infections on the blood parameters of naturally infected goats is scarce. This study aims to (1) identify the presence of hemotropic *Mycoplasma* in goats from both suburban and rural regions of Bosnia and Herzegovina using polymerase chain reaction (PCR), and (2) assess any potential correlation between hemoplasmosis and the hematological parameters of the infected goats.

Materials and Methods

Blood sampling

Blood samples were gathered from goats situated on farms in Bosnia and Herzegovina, specifically from farm A (43°53'47.4"N 18°23'28.7"E) in a suburban area ($n = 10$) and farm B (44°03'33.2"N 18°27'09.0"E) in a rural location ($n = 10$) (Fig. 1). Blood samples were transported in portable refrigerators at a temperature of 4°C to the University of Sarajevo—Veterinary Faculty, where they underwent additional processing. The study was conducted during the winter of 2019. Throughout the examination period, temperatures in both areas fluctuated between −10°C and −17°C, while the annual average precipitation was 1,000 mm for the year 2019. These particular areas were chosen due to the presence of pastoralist farmers who co-graze their livestock with wildlife, increasing the likelihood of pathogen transmission between species. The age of sampled animals ranged from 6 months to 7 years.

Hematology and serum biochemistry analysis

For hematological analyses and molecular diagnostics, blood samples were gathered in vacutainers that contained the anticoagulant ethylenediaminetetraacetic acid (EDTA). Hematological parameters, including red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), Reticulocyte count (RETIC), white blood cell (WBC), as well as cell differentiation of WBC and determination of absolute values of Neutrophils, Lymphocytes, Monocytes, Eosinophils, Basophils; platelet count (PLT), and mean platelet volume (MPV) was determined using automatic analyzer (ProCyte Dx, IDEXX, USA). Blood vacutainers without anticoagulant were allowed to coagulate, centrifuged at 3,000 rpm for 10 minutes, and measured using standard procedures with the Catalyst One™ Chemistry Analyzer (IDEXX Laboratories, Netherlands). The concentration of glucose (GLU), albumin (ALB), globulin (GLOB), albumin globulin ratio, total protein (TP), creatinine (CREA), urea (UREA), ratio blood urea nitrogen and creatinine (BUN/CREA), total bilirubin (TB), cholesterol (CHOL), calcium (Ca), phosphorus (PHOS), as well as the activity of alanine aminotransferase (ALT), alkaline phosphatase (ALKP), gamma-glutamyl transferase (GGT), amylase (AMYL), and lipase (LIPA) had been determined.

DNA isolation

Total genomic DNA from 100 µl of anticoagulated whole blood was isolated using DNeasy® Blood and Tissue kit (Qiagen, Germany) following the manufacturer's guidelines and was eluted from the column using 100 µl of Tris-EDTA buffer. DNA concentration was estimated using Qubit® fluorimeter (ThermoFisher Scientific, USA) according to the manufacturer's instructions. DNA was stored at −20°C until PCR testing.

Confirmation of Mycoplasma spp. by polymerase chain reaction

We amplified 600 bp fragments of 16S rRNA of *Mycoplasma spp*. using the following primers: HBT-F: 5'-ATA CGG CCC ATA TTC CTA CG-3' and HBT-R: 5'-TGC TCC ACC ACT TGT T CA-3' (Criado-Fornelio *et al.*, 2003). The composition of each 25 µl reaction mixture was as follows: 12.675 µl of sterile distilled water, 0.125 µl of GoTaq® DNA polymerase (Promega, USA), 5 µl of Green Reaction Buffer (Promega, USA), 0.2 µl of dNTPs, 1 µl of each primer, and 5 µl of template DNA. These reactions were prepared in batches of 10. The conditions of the PCR reaction were as follows: initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a final extension step at 72°C for 7 minutes. All samples were tested three times. Positive and negative controls were utilized to confirm the results. PCR products were examined through electrophoresis on 2% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Germany). Positive samples were forwarded to a commercial laboratory (LGC Genomics GmbH, Germany) for sequencing using

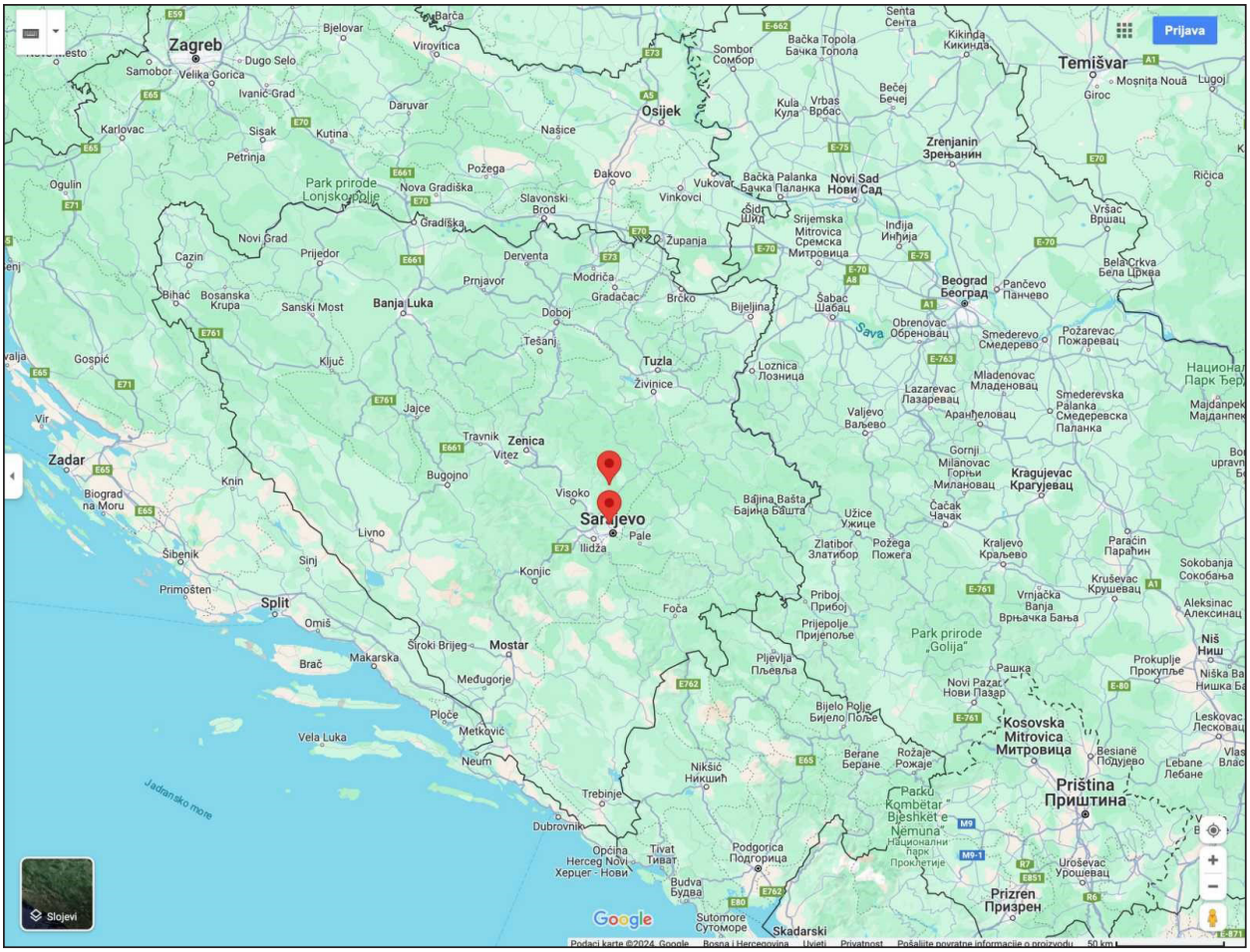

Fig. 1. Map of Bosnia and Herzegovina showing the location of the two sampling locations (Farm A and Farm B). The figure was generated and modified using https://www.google.com/maps.

amplification primers. The representative sequence of seven identical clones was deposited to GenBank under the number PP359532.

Phylogenetic analysis of Mycoplasma sequences

Obtained sequences and relevant phylogenetic data from GenBank were aligned using MUSCLE (Edgar, 2004). A phylogenetic tree demonstrating the relationship of sequences obtained in this study and known *Mycoplasma* sequences was created by the Maximum likelihood method based on the General Time Reversible model (Nei and Kumar, 2000) and considering among-site variation by employing a four-category discrete approximation of a Γ distribution with a fraction of invariable sites using MEGA11 (Tamura *et al.*, 2021). Bootstrap support values were assessed by 1,000 bootstrap replications.

Statistical analysis

All results are presented as means ± standard deviation (SD). A *p*-value of < 0.05 was deemed statistically significant. Comparisons between groups were made using the unpaired-sample Student's *t*-test. Statistical analyses were performed using the Mini Tab software (Version 19/2020).

Results

Using a PCR assay 35% (7 of 20) animals tested positive for *Mycoplasma* infection. Sequence comparisons revealed that the 16S rRNA fragments were clonal as they were 100% identical. Comparisons with the nucleotide collection database available from GenBank showed that the sequences were 100% identical to *M. ovis* (AF338268) as well as to several other *M. ovis* sequences including sequences originating from goats (KU983745), sheep (EU16551, ON885266), wildcat (ON202709), and human (KF313922). On a phylogenetic tree (Fig. 2.), these sequences formed a separate cluster with *M. ovis*.

The results of our study revealed significant hematological differences for some parameters between goats infected with *M. ovis* and those without the infection. Although the obtained values for the examined parameters within the reference values,

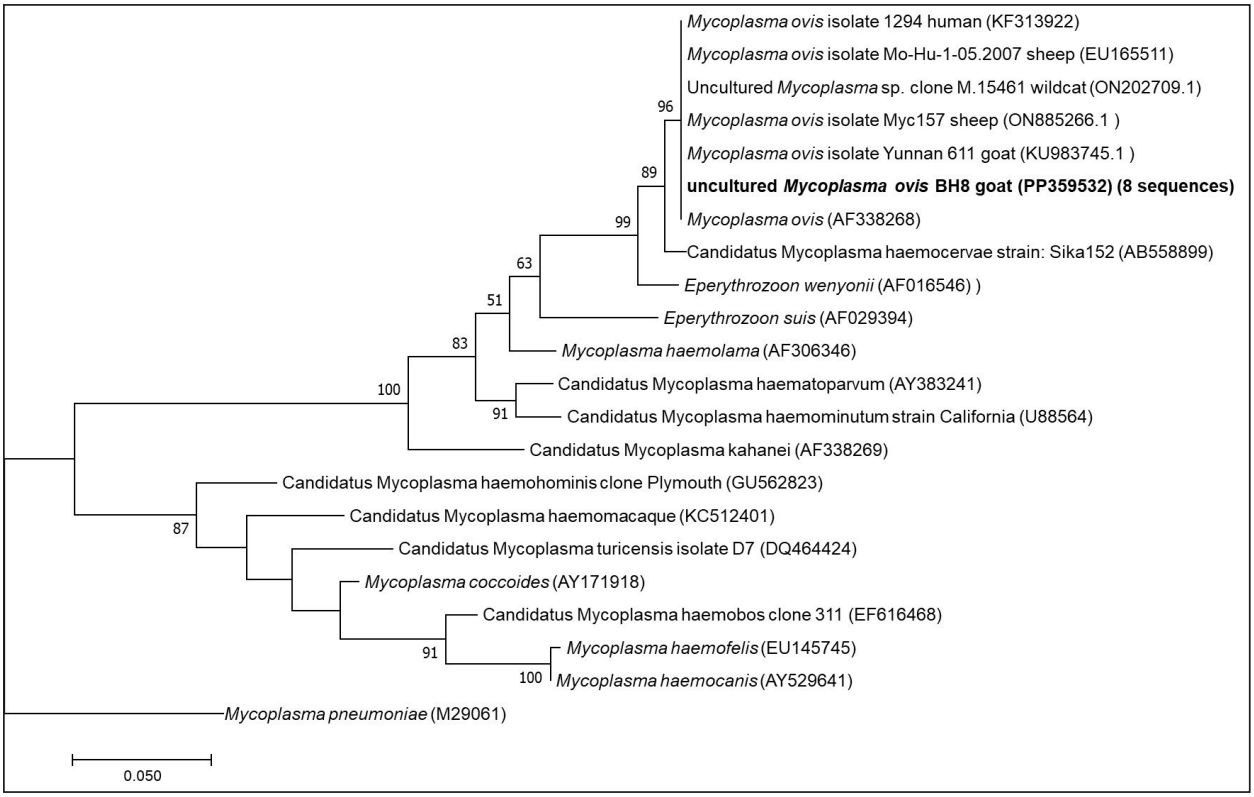

Fig. 2. ML phylogenetic tree showing the phylogenetic position of sequences obtained in this study and other *mycoplasma*s. Only bootstrap values of >75% are shown. The *M. ovis* sequences detected in this study are shown in bold.

indicated that none of the goats became anemic, statistical differences were still observed among the groups of animals studied.

Discussion

This research examines the occurrence of hemotropic *M. ovis* in randomly chosen goat herds in Bosnia and Herzegovina. Since *M. ovis* is an obligate epicellular bacterium that does not remain stable under laboratory conditions and is easily damaged by drying or disinfectants, it cannot be grown in cell-free media (Windsdor, 2022). Before the development of PCR assays, estimating parasitemia in haemoplasma infection relied on subjective blood smear examination, demanding a high level of expertise (Paul *et al.*, 2020). The effectiveness of blood film assessment in terms of sensitivity and specificity is also limited (Wangai *et al.*, 2011). Additionally, studies have noted that the degree of bacteremia observed in goats via microscopy is lower compared to that in sheep; this observation has been confirmed through molecular techniques (Johnson *et al.*, 2016). The use of more advanced and accurate molecular techniques, such as conventional PCR assays and quantitative PCR assays, has greatly improved the ability to diagnose hemoplasma infections in various host species.

There are few reports on the hematological and biochemical parameters of small ruminants with haemotropic mycoplasmosis. Some authors report neutrophilic left shift, monocytosis, and lymphocytosis (Souza *et al.*, 2019). Besides, there are reports on *M. ovis* infection accompanied by significant biochemical alterations, such as hyponatremia, hypocalcemia, hypoalbuminemia, reduced protein levels, and a simultaneous rise in serum creatinine, indirect bilirubin, GGT, AST, ALP, and BUN (Souza *et al.*, 2019).

It is possible that an untested concurrent infection could have contributed to the observed lower values in HCT and hemoglobin levels in goats that tested negative for *M. ovis*. Another study found that 73% of the animals in their cohort had coinfections (Paul, 2020). In addition to this synergistic relationship, hemotropic *mycoplasma*s, acting independently, have been shown to induce severe anemia (Adduci *et al.*, 2022).

Goats negative for *M. ovis* may be experiencing different types of immune responses or activation of alternate immune mechanisms, which could lead to higher baseline WBC counts. Additionally, goats testing positive for *M. ovis* may be experiencing immune suppression or a subdued immune response. This can occur due to the pathogen's capacity to evade or suppress the host's immune system, particularly the inflammatory responses (Askar *et al.*, 2021).

It is essential to highlight that individual variation and environmental factors significantly influence immune responses. The observed significant increase in neutrophil counts in goats that tested negative for *M. ovis* (Table 1), coupled with lower levels in goats that tested positive for the pathogen aligns with the previously discussed variations in white blood cell counts, suggesting a complex interaction between *M. ovis* infection and the host's immune response.

A study indicates that in a goat, co-infection with Ehrlichia ewingii and hemotropic *Mycoplasma* resulted in macrocytic hypochromic anemia characterized by anisocytosis, macrocytosis, basophilic stippling, elevated levels of AST, CK, and GGT, and reduced ALP activity (Meichner *et al.*, 2015). Likewise, reports have indicated co-infections of erythrocytes with hemotropic *Mycoplasma* and *Anaplasma* species in goats (Ait Lbacha *et al.*, 2017). Concurrent infection with other tick-borne hemopathogens heightens the vulnerability of animals to hemotropic *mycoplasma*s. (Varanat *et al.*, 2011). This occurs because the presence of multiple co-infecting pathogens induces complex and varied responses, facilitating synergy and more effective colonization within the host (Baneth, 2014).

The hypoglycemia found in the blood samples (Table 2) is believed to have a dual potential cause: (1) the utilization of circulating glucose by *M. ovis* within the host, resulting in genuine hypoglycemia; and (2) the consumption of glucose *in vitro* by *M. ovis* present within the collected blood sample may cause artifactually decreased blood glucose levels (Burkhard and Garry, 2004; Almy *et al.*, 2006). Studies on *M. suis* suggest that the severity of hypoglycemia appears to correspond to the level of haemoplasma bacteremia (Tasker *et al.*, 2009). Furthermore, two studies specifically highlight significant hypoglycemia in the absence of any related clinical signs (Burkhard and Garry, 2004, Humann-Ziehank and Ganter, 2012).

Changes in serum biochemical parameters with goats positive for *M. ovis* showed values lower than the reference range for total protein concentration in both groups of animals, as well as lower concentrations of creatinine and cholesterol, higher concentration of TBIL, and lower activity of ALT and GGT (Table 2).

Mycoplasma infections are among significant goat diseases and cause notable losses in many European countries, the United States, as well as Australia and Africa (DaMassa *et al.*, 1992).

The actual distribution of *M. ovis* remains uncertain as there are limited studies documented in the literature. Previous investigations suggest that the infection is widespread (Paul at al., 2020). In Europe, *M. ovis* has been detected in several countries including Cyprus, France, Germany, Hungary, Ireland, Norway, and the

Table 1. Values of hematological parameters in goats infected with *M. ovis* and non infected goats.

Hematological parameter	*Mycoplasma* infected goats	*Mycoplasma* non-infected goats
RBC	18.68 ± 3.94	17.42 ± 3.12
HCT*	34.26 ± 3.05	27.41 ± 4.86
HGB*	101.00 ± 11.75	90.76 ±11.18
MCV	18.87 ± 3.39	16.17 ±4.16
MCH	5.49 ± 0.49	5.27 ± 0.52
MCHC*	29.54 ± 2.79	34.03 ± 6.35
RETIC	3.94 ± 2.55	3.68 ± 2.31
WBC*	11.36± 2.86	14.58 ± 3.37
%NEU	36.66 ± 10.99	42.04 ± 12.22
%LYM	50.17 ± 8.94	46.88 ± 12.57
%MONO	9.75 ± 6.66	8.27 ± 6.15
%EOS	2.57 ± 1.28	1.94 ±1.46
%BASO	0.84 ± 1.92	0.88 ± 1.86
NEU*	4.30 ± 2.08	6.11 ± 2.18
LYM	5.58 ± 1.12	6.82 ± 2.43
MONO	1.11 ± 1.72	1.26 ± 1.12
EOS	0.3 ± 0.03	6.82 ± 2.43
BASO	0.07 ± 0.17	0.13 ± 0.26
PLT	422.43 ± 90.89	457.48 ± 123.53
MPV	7.89 ± 0.32	7.98 ± 0.75

Value represents the mean ± standard deviation of two groups of goats; * Significant differences in parameter values among goats infected with *M. ovis* and non-infected group ($p < 0.05$).

Table 2. Values of biochemistry parameters in goats infected with *M. ovis* and non infected goats.

Biochemistry parameter	*Mycoplasma* infected goats	*Mycoplasma* non-infected goats
GLU*	2.51 ± 0.13	2.98 ± 0.33
CREA	50.71 ± 9.83	56.62 ± 19.78
UREA	6.04 ± 2.33	4.94 ± 1.8
BUN/CREA	29.57 ± 11.1	22.76 ± 9.18
PHOS	1.56 ±0.49	1.79 ± 0.52
CA	2.3 ± 0.24	2.33 ± 0.14
TP	86.14 ± 5.64	81.29 ± 7.4
ALB*	29.71 ± 1.6	26.38 ± 2.91
GLOB	56.57 ± 4.5	54.59 ± 5.41
ALP/GLOB*	0.54 ± 0.05	0.49 ± 0.07
ALT	11.71 ± 7.48	13.14 ± 9.43
ALKP	147.86 ± 123.55	263 ± 217.73
GGT	58.57 ± 12.58	53.05 ± 11.37
TBIL	9.29 ± 1.7	8.57 ± 2.31
CHOL	1.54 ± 0.66	1.97 ± 0.71
AMYL	26.14 ± 4.71	25.43 ± 8.69
LIPA*	196.86 ± 53.82	151.48 ± 56.55

Value represents the mean ± standard deviation of two groups of goats; * Significant differences in parameter values among goats infected with *M. ovis* and non-infected group ($p < 0.05$).

UK (Stuen, 2016). This research is, to the best of the authors' knowledge, the first molecular detection of *M. ovis* in goats in Bosnia and Herzegovina as well as the first report in this geographical region. This research represents the inaugural confirmed identification of hemoplasma infection in goats from Bosnia and Herzegovina, achieved through a PCR-based assay. The findings from this study aim to enhance awareness among farmers and veterinarians, leading to improved health management practices and overall goat herd well-being. Including more comprehensive immune response markers in future studies could elucidate the reasons behind the observed discrepancies in WBC counts among different groups.

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None.

Conflict of interest

The authors declare that there is no conflict of interest.

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Authors' contributions

N.H., L.P., J.Š.: Conceptualization, Methodology, Software; L.P., H.P.F., B.S.B: Project administration; L.P.: Data curation, Supervision, Validation; A.L, N.H, L.P: Writing- Original draft preparation; A.L., N.H., L.P., J.Š.: Visualization, Investigation.

Data availability

All data supporting the findings of this study are available within the manuscript.

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