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Original Article

Platelet Indices and Hemoglobin, Albumin, Lymphocyte, and Platelet (HALP) Score Alterations in Sheep with Molecularly Confirmed *Theileria* spp. Infection

Vahid Dokhaei [1], *Mahmood Ahmadi-Hamedani [2], *Mohammad Reza Salimi Bejestani [1]

1. *Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran*
2. *Department of Clinical Sciences, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran*

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***Correspondence Emails:**
ahmadi.hamedani@semnan.ac.ir
msalimi@semnan.ac.ir

Abstract

Background: The hemoglobin (Hb), albumin (ALB), lymphocyte (LYM), and platelet (PLT) components of the HALP score, a human-derived immune-nutritional index, are currently being investigated in the veterinary field. We aimed to evaluate and compare the HALP score and platelet indices (PI) in *Theileria*-infected and non-infected sheep.

Methods: One hundred sheep from different farms in Semnan Province were screened for the presence of *Theileria* between June and August 2023 using microscopy and molecular-based methods. Hematocrit (Hct), Hb, red blood cell (RBC), LYM, PLT, plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), and ALB were measured. The HALP score was calculated by multiplying Hb in ALB by LYM and dividing by PLT.

Results: Microscopic and PCR analyses identified 21 and 27 positive samples for *Theileria* species, respectively. Results showed *T. ovis* in 52%, *T. lestoquardi* in 26%, *T. annulata* in 11%, and a co-infection of *T. ovis* and *T. lestoquardi* in 11% of the samples. The PLT and PCT showed significant differences in sheep infected with *T. lestoquardi*. A significant difference was also observed in PDW between sheep infected with *T. ovis* and *T. lestoquardi* compared to those infected with *T. annulata*. Statistical differences in the HALP score were noted for *T. lestoquardi* ($P = 0.03$), *T. annulata* ($P = 0.01$), and co-infections of *T. ovis* and *T. lestoquardi* ($P = 0.05$) versus *T. ovis*.

Conclusion: PLT indices (PI) and HALP scores can improve diagnostic processes, and future studies should use them alongside other methods for diagnosing *Theileria* species.

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Introduction

Theileria species are apicomplexan tick-borne parasites that significantly constrain the global livestock industry, causing economic losses ranging from 5% to 25% (1-3). In Iran, *T. lestoquardi* (the agent of acute form) and *T. ovis* are the most prevalent species affecting small ruminants (4, 5). Although theileriosis is known for its systemic symptoms, such as fever, jaundice, and anemia, it also has a drastic effect on the blood profile. In particular, the acute stage of the disease significantly affects the coagulation cascade and platelet count, which may lead to mucosal hemorrhage and/or petechiae (6, 7). In experimental infection with *T. lestoquardi*, a decline in hemoglobin and changes in white blood cells are observed (8). Laboratory findings of theileriosis include hypoalbuminemia, increased liver enzyme activity, and renal failure (9). Changes in the cardiovascular system and increased certain enzymes, such as cardiac troponin I, are also observed (10).

Platelets are not only essential for clot formation but also serve as key regulators of inflammation and immune response by interacting with leukocytes and releasing chemotactic cytokines (11).

In *Theileria*-infected animals, changes in platelet count (PLT) and indices such as Plateletcrit (PCT) and Mean Platelet Volume (MPV) have been frequently observed, correlating with the severity of clinical symptoms. Since MPV and Platelet Distribution Width (PDW) reflect functional changes and platelet activation, evaluating these indices through a Complete Blood Count (CBC) provides a cost-effective method to assess the inflammatory status of the host (12).

A novel approach to evaluating the host's response is the use of the HALP score, a composite of hemoglobin (Hb), albumin (ALB), lymphocyte (LYM), and PLT. Developed initially to predict prognosis in various systemic inflammatory conditions, the HALP score integrates immune status (lymphocytes and platelets) with the physiological state (Hb and ALB) (13, 14).

In the context of theileriosis, the given score is particularly relevant because the pathology simultaneously causes anemia, manifested by reduced hemoglobin concentrations, as well as inflammation-induced changes in plasma protein concentrations, most notably ALB, and significant changes in leukocytic and thrombocytic lineages. Therefore, the HALP score could offer comprehensive insight into the immunoinflammatory balance in infected sheep that single parameters might fail to capture (14, 15).

Despite extensive research on the molecular diagnosis and prevalence of *Theileria* species (16-23), limited information is available regarding the diagnostic or prognostic value of platelet indices and the HALP score in small ruminant theileriosis. Therefore, this study aimed to evaluate and compare the HALP score and platelet indices (PI) in *Theileria*-infected and non-infected ovine.

Materials and Methods

Sampling

One hundred sheep from different farms in Semnan Province, central Iran were screened for *Theileria* species between June and August 2023 using microscopy and molecular-based methods. Semnan Province is located between 34°13' and 37°20' N latitude and 51°51' and 57°03' E longitude, covering a total area of 97,491 km².

Two types of tubes were used in the sample: 1) tubes with no anticoagulant to determine biochemical profiles, and 2) tubes with EDTA to identify hematological parameters.

These experiments were conducted according to established animal welfare guidelines and approved by the Animal Research Ethical Committee of Semnan University of Veterinary Medicine (Ethics Code: IR.SU.REC.1401.139).

Microscopic observation

Blood smears were fixed with methanol and stained with Wright-Giemsa stain at a 5% dilution in buffer solution. Subsequently, they were examined under a light microscope at 100x magnification using immersion oil. Two experienced observers evaluated each smear across multiple fields for *Theileria* piroplasms. A sample was considered negative for *Theileria* spp. only if neither observer detected piroplasms in any of the examined fields.

DNA extraction and PCR analysis

Genomic DNA was extracted from blood samples using the phenol-chloroform method, followed by genus identification of *Theileria* using the Polymerase Chain Reaction (PCR) technique. PCR reactions were performed in a 20 µL volume containing 2X PCR master mix, forward and reverse primers, DNA template, and sterile distilled water. The thermal profile included 34 cycles, with an expected product size of 686 bp. A portion of the 18S rRNA gene was amplified using P3/P4 primers. The expected PCR product sizes were 420–436 bp for *Theileria* and 389–402 bp for *Babesia*. The annealing temperature for these primers was 54 °C. Due to the inability to distinguish these two species using semi-nested PCR and the 18S rRNA gene, the *Tams1* gene was used. Two forward primers (P7 for *T. lestoquardi* and P8 for *T. annulata*) and one shared reverse primer (P9) were designed for this purpose (17) (Table 1).

Table 1: The oligonucleotide primers for identification *Theileria* and *Theileria* spp. used in this study

Primer name	Gene name	Reference	Sequence number	PCR production (bp)
P1	*Ovine β-actin*	U39357	5´-ATCACTGCCCTGGCACCCAG-3´	686
P2			5´-CTGGAGACACTGAGCAGTCTG-3´	
P3		AF081135	5´-CACAGGGGAGGTAGTGACAAG-3´	*Theileria* spp. 426-430
		GU726904		
P4	*18S rRNA*	AY260178	5´-CTAAGAATTTCACCTCTGACA-3´	*Babesia* spp. 389-402
P5		AF081135	5´-CTTTACGAGTCTTTGCATTG-3´	*T. ovis* 228
P6		GU726904	5´-ATTGCTTGTGTCCCTCCG-3´	*T. lestoquardi* 237
P7		AJ006448.1	5´-GTGCCGCAAGTGAGTCA-3´	*T. lestoquardi* 760
P8	*ms1*	AB917302.1	5´-ATGCTGCAAATGAGGAT-3´	*T. annulata* 780
P9		AJ006448.1	5´-GGAATGATGAGAAGACGATGAG-3´	Common reverse primer

Measurement of clinicopathological parameters

An automatic veterinary cell counter (Nihon Kohden, Japan) was calculated hemogram (Hct, Hb, RBC, and RBC-related indices), leukogram (leukocyte counts and differential leukocyte counts), and thrombogram (PLT, PCT, MPV, and PDW). An autoanalyzer (917 Hitachi, Japan) was measured the serum ALB concentration using a commercial biochemical kit (Delta Darman Part, Tehran, Iran).

Measurement of HALP score

The HALP score is obtained from the following formula (Chen et al., 2015): HB (g/L) × ALB (g/L) × LYM ($\times 10^9$/L) ÷ PLT ($\times 10^9$/L)

Statistical analyzes

For data analyzes, the Shapiro-Wilk test was used to assess the data's normality. If the data distribution was normal, the student's *t*-test was used to compare PCR-positive and PCR-negative animals. For more than two groups, one-way ANOVA was utilized. If the data did not follow a normal distribution, the median and interquartile range (IQR) were measured between groups using the Kruskal Wallis test. $P < 0.05$ were considered statistically significant. McNemar's chi-square test was used to

compare the data from the microscopic examination with the PCR method.

Results

Out of 100 sheep blood samples, 79 were negative, and 21 were positive (Fig. 1).

Among 100 sheep blood samples, 27 positive and 73 negative samples were reported using PCR (Fig. 2). PCR's sensitivity and specificity for diagnosing *Theileria* spp. were 71% and 93%, respectively (Table 2). Of the 27 positive samples, 14 samples were identified as *T. ovis* (52%), 7 samples as *T. lestoquardi* (26%), 3 samples as *T. annulata* (11%), and 3 samples showed co-infection with *T. lestoquardi* and *T. ovis* (11%).

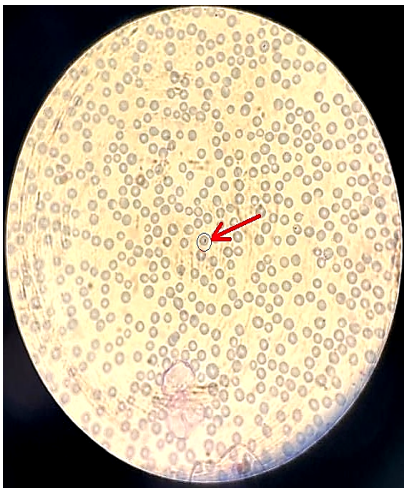

Fig. 1: Piroplasm of *Theileria* spp. in red blood cells (Original)

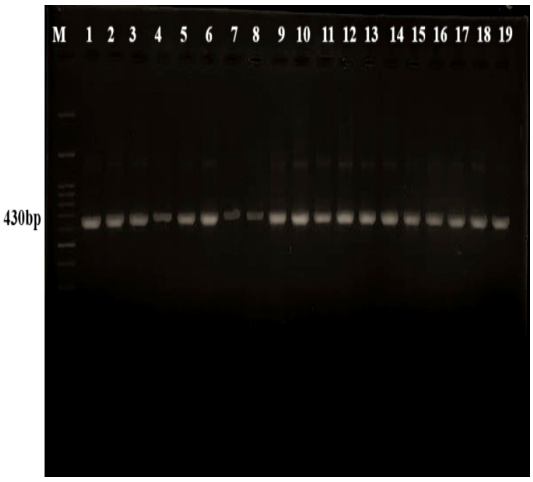

Fig. 2: Agarose gel electrophoresis of amplicons of *Theileria* spp. *Lane* M revealed 100bp DNA ladder, Lanes 1-19 indicated positive animals

Table 2: Agreement between PCR method and blood culture in the diagnosis of theileriosis

	PCR (+)	PCR (-)	Total
Microscopy (+)	16	5	21
Microscopy (-)	11	68	79
Total	27	73	100
Sensitivity: 71%		Specificity: 93%	

The results obtained from comparing PI between uninfected and *Theileria* infected sheep showed that the PLT and PCT in the *Theileria* infected group was significantly decreased compared to the uninfected group (P = 0.026 and 0.050). Regarding the MPV and PDW, although the mean rank of MPV and PDW were higher in the *Theileria* infected group, these differences were not statistically significant (Table 3).

Table 3: The comparison of PLT, PCT, MPV, and PDW in uninfected and *T.* infected sheep

	PLT (× 10^3/µl)		PCT (%)		MPV (fl)		PDW (%)	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
n	63	26	62	26	65	26	70	27
Mean	898.58 ± 249.02	544.42 ± 173.98	0.52 ± 0.17	0.42 ± 0.14	6.23 ± 0.55	6.55 ± 0.99	13.69 ± 1.86	14.74 ± 2.83
***P* value**	0.026		0.050		0.394		0.071	

Furthermore, while RBC, Hb, HCT, LYM, and ALB levels were lower in the *Theileria* infected sheep, only the decrease in Hb reached statistical significance ($P<0.05$; Table 4).

Table 4: The comparison of RBC, Hb, HCT, Lymphocytes, and ALB in uninfected and *T.* infected sheep

	RBC (× 10^6/µl)		Hb (g/dl)		HCT (%)		Lymphocytes (%)		ALB (g/dl)	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
n	64	26	73	26	73	26	73	27	71	24
Mean rank	47.16	41.42	53.45	40.31	52.16	43.92	51.32	48.23	48.80	45.65
***P* value**	0.345		0.045		0.209		0.641		0.628	

The results of comparing PI in sheep infected with different *Theileria* species are presented in Table 5. The lowest PLT and PCT levels were observed in sheep infected with *T. lestoquardi*. A statistically significant difference was found between sheep infected with *T. lestoquardi* and other species. The highest PDW level was recorded in sheep infected with *T. annulata*. A statistically significant difference was found between sheep infected with *T. annulata* and sheep coinfected with *T. ovis* and *T. lestoquardi* (Table 5).

Table 5: Comparing PI in sheep infected with different species of *Theileria*

Theileria spp.	PLT (× 10^3/µl)	PCT (%)	MPV (fl)	PDW (%)
T. ovis	913.71 ± 177.04^a	0.57 ± 0.12^a	6.28 ± 0.54^a	14.14 ± 1.02^{ab}
T. lestoquardi	584.33 ± 48.78^b	0.37 ± 0.05^b	6.45 ± 0.85^a	13.53 ± 1.69^{ab}
T. anulata	1018.33 ± 122.41^a	0.59 ± 0.06^a	5.83 ± 0.15^a	15.13 ± 0.75^a
T. ovis-T. lestoquardi	980.80 ± 106.71^a	0.62 ± 0.07^a	6.40 ± 0.53^a	13.00 ± 0.87^b

The HALP score in *Theileria* infected sheep showed a downward trend compared to the uninfected group. However, this difference was statistically non-significant, although it showed a marginal trend toward significance ($P=0.055$). The mean rank of HALP score in sheep infected with *T. annulata* was lower than in other *Theileria* species (Table. 6).

Table 6: The comparison of HALP score in sheep infected with various species of *Theileria*

Theileria spp.	HALP Score				
	Median	IQR	Sig.		
T. ovis	14.155^a	(10.67-19.01)	0.03	0.01	0.05
T. lestoquardi	13.97^b	(12.61-15.61)			
T. anulata	8.52^b	(4.55-11.08)			
T. ovis-T. lestoquardi	11.76^b	(11.25-14.38)			

Discussion

The present study evaluated the significance of platelet-related indices and the HALP score in sheep naturally infected with various *Theileria* species. In examining PI between the uninfected and *Theileria* infected sheep and differentiating between various species of the causative agent, PLT and PCT in the uninfected sheep was higher than in *Theileria* infected sheep. The PDW in the *Theileria* infected sheep was greater than that in the uninfected sheep. Still, since this difference was insignificant, it cannot be said that *Theileria* causes a difference in platelet size. It indicates that thrombocytopenia has occurred due to the

destruction and consumption of platelets, and there is no disruption in the platelet production process. The HALP score in *Theileria* infected sheep decreased compared to uninfected sheep and among the different species of *Theileria*, *T. annulata* showed the lowest score of HALP, indicating that *Theileria* causes a poorer immunonutritional status in infected sheep. Given the significant impact of this disease on Iranian animal husbandry (24), several diagnostic investigations have been conducted; however, no study has investigated PI and HALP score in *Theileria* infected sheep. The present study is the first to examine PI and the HALP score in *Theileria* infected sheep in Iran.

The dominance of *T. ovis*, as determined within this study, is well supported by multiple molecular studies conducted within Iran. For example, in the province of Zabol, in the southwest of Iran, and across multiple provinces, the most prevalent species is *T. ovis* (25-27). However, variations do exist within specific areas, for example, within the northern provinces, the prevalence of *T. lestoquardi* is greater (28).

Apart from species identification, the importance of theileriosis lies in its hematological changes. To appreciate this, a comparison of these modifications with those in similar diseases in other species would be pertinent. For example, as in canine babesiosis and ehrlichiosis, anemia and thrombocytopenia are prominent features of theileriosis (29-32), which justifies evaluating complex hematological indices, such as the HALP score, in the present study. In a study, dogs affected by theileriosis exhibited clinical signs of pale mucous membranes, a tendency to bleed, and lethargy. Laboratory examinations revealed thrombocytopenia, anemia, and myelofibrosis. Following treatment, the anemia and thrombocytopenia diagnosed at presentation normalized. At the end of the treatment, hematocrit, PCT, and white blood cell count increased (33). Another study examining PI in dogs infected with *Babesia* reported that PLT in infected dogs was lower than in healthy dogs, while MPV in infected dogs increased. The decrease in platelet numbers during intracellular parasitic diseases is associated with forming platelet aggregates and giant platelets, leading to abnormal blood clotting and coagulopathy (34). The findings of this research are consistent with the results of the present study.

The present study reports elevated MPV and PDW, suggesting increased platelet activity and systemic inflammation (35). A significant reducing the PLT and PCT indicates excessive platelet consumption (36). In thrombocytopenia, the bone marrow is activated, increasing MPV due to the enhanced production of giant cells (37). PCT predicts bleeding risk in patients with thrombocytopenia (38). PDW increases in the presence of platelet anisocytosis (39). With the decrease in platelet synthesis, immature platelets become larger and more active, increasing MPV (40).

In the present study, a reduction in PCT was observed despite the increase in MPV in *T.* infected sheep (due to a decrease in PLT). Theileriosis causes changes in hematologic parameters, such as a reduction in RBC, Hb concentration, and HCT levels, which is consistent with the results of the present study (41-43). Theileriosis, through increased oxidative stress, leads to heightened vulnerability of RBC due to membrane lysis and a decrease in Hb concentration.

In the study (44), no significant difference was observed in the number of lymphocytes, leukocytes, and granulocytes between sick and infected sheep. However, in the study by Al-Hamidhi et al. (45), a significant reduction in WBCs, monocytes, and lymphocytes was observed.

Various studies have also reported that in *Theileria*-infected, levels of total protein and ALB are reduced (41, 46, 47). Different species of *Theileria* lead to a decrease in protein concentration, which is attributed to hypoalbuminemia resulting from decreased ALB syn-

thesis. The role of reduced dietary protein intake and diarrhea should not be overlooked. Additionally, hypoalbuminemia may occur due to the accumulation of protein-rich fluid in the extravascular space resulting from lymphatic gland damage (48).

The present study observed decreased RBC, HCT, Hb, ALB, and LYM. Anemia, weakness, anorexia, gastrointestinal disorders, and lymphadenopathy have been reported in infections with theileriosis (6). The HALP score is an integrated criterion for immunonutritional and general host condition. *Theileria*-infected sheep had significantly lower HALP scores than the healthy group. These changes are like those seen in other pathological conditions characterized by nutritional and inflammatory disorders (49). However, it is important to consider the limitations of this comparison, as the underlying causes may differ significantly between the conditions.

Conclusion

Infection in sheep with various *Theileria* species, particularly *T. lestoquardi*, leads to significant alterations in PI, notably PLT, and PCT. Moreover, *T. annulata* is associated with a reduction in the HALP score. Future studies incorporate these parameters alongside existing diagnostic methods for identifying *Theileria* and its various species.

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Conflict of Interests

The authors declare no conflict of interest.

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