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Evaluation of clinicopathological changes in haemoprotozoan affected small ruminants in and around Bangalore

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Abstract
The present study aimed to conduct a clinicopathological evaluation of haemoprotozoan-affected small ruminants. Small ruminants presented to the Veterinary College Hospital, Hebbal, Bengaluru, with classical clinical signs suggestive of haemoprotozoan disease, such as anorexia, high temperature, lymphadenopathy, dullness, pale mucous membranes, icterus, emaciation, diarrhoea, and a history of tick infestation. Blood samples were collected from suspected animals, and 10 of them tested positive for haemoprotozoa in blood smear examinations (6 for *Theileria* and 4 for *Babesia*). These findings were further confirmed using polymerase chain reaction (4 for *Theileria*, 4 for *Babesia*, and 2 for both *Theileria* and *Babesia* organisms). When compared to healthy animals, infected animals showed a significant increase in physiological parameters such as temperature, respiratory rate, and heart rate, along with a significant decrease in rumeno-reticular motility. Haemato-biochemical parameter analysis of blood samples from positive animals revealed a significant decrease in haemoglobin, total erythrocyte count (TEC), packed cell volume (PCV), and thrombocyte count. There was also a significant increase in leucocyte count. Biochemical analysis indicated a significant decrease in total protein, albumin, globulin concentration, and a significant increase in blood urea nitrogen level (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase, and total bilirubin in animals with haemoprotozoa infection compared to their healthy counterparts.

Keywords: Clinicopathological, haemoprotozoa, *Theileria*, *Babesia* and haemato-biochemical

Introduction
Vector-borne haemoprotozoans constitute a diverse group of single-celled eukaryotic organisms transmitted by blood-feeding invertebrates. These parasites possess the capability to cause severe diseases in various hosts, impacting livestock, wildlife, and companion animals. (Egan *et al*., 2021) [6]. *Babesia* and *Theileria* species are the important tick-borne haemoprotozoan parasites that infect small ruminants in different regions, including tropical and subtropical areas (Onyiche *et al*., 2019) [17]. Hemoprotozoan parasites pose a serious threat to the livestock population in terms of mortality, reduced milk yield, lowered draft power and meat production (Maharana *et al*., 2016) [15].

The most common technique for diagnosing clinical babesiosis and theileriosis in small ruminants is the Giemsa staining technique (Razmi *et al*., 2002) [20]. Polymerase chain reaction (PCR) is the most commonly used molecular technique for detecting piroplasms in recent years compared to conventional blood smear examination (Gunes *et al*., 2016) [8]. In the field of haematology, the primary clinical indicator linked with haemoprotozoan parasites is anaemia. Anaemia is described as an abnormal condition marked by a reduction in haematocrit (packed cell volume), the quantity of erythrocytes (Red Blood Cells), and/or haemoglobin (Katsogiannou *et al*., 2018) [13]. Serum biochemical analysis indicated a notable elevation in ALT, AST, creatinine, blood urea nitrogen, and total bilirubin levels. Simultaneously, there was a considerable reduction in total plasma protein and albumin levels (Mahmoud *et al*., 2019 and Eliwa *et al.* 2021) [16, 7].

Studying the clinicopathological alterations linked with haemoprotozoan infection is crucial for the early identification and proper treatment of the disease. The elevated cost of treatment, diminished productivity, and potential fatality in severe cases make haemoprotozoan infection a significant economic burden on small ruminants in India.

Materials and Methods

Small ruminants presented to the Veterinary College Hospital, Bengaluru, with typical clinical signs indicative of haemoprotozoan disease such as anorexia, elevated temperature, lymphadenopathy, dullness, pale mucous membranes, icterus, emaciation, diarrhoea, and a history of tick infestation were included in this study. Blood smear examinations were performed to identify piroplasms associated with haemoprotozoan infections, and subsequent confirmation was achieved through PCR.

A thorough clinical examination was conducted for each animal, adhering to the standard methods outlined by Kelly (1984) [14].

Group I (Control group) (N=6): Consisted of apparently healthy animals that tested negative for haemoprotozoa in both blood smear examinations and PCR.

Group II (N=10): Animals that were identified as positive for haemoprotozoa through both blood smear examinations and PCR.

Blood smear examination: Blood smear was made using blood collected from the peripheral circulation such as ear vein, fixed with absolute methanol (30 seconds to 1 minute), stained with 10% Giemsa stain (30 mins) and examined under oil immersion microscope to observe intraerythrocytic forms of piroplasms and categorized into *Babesia* and *Theileria* organism. The parasites were identified according to the characters described by Soulsby (1982) [23].

Hematobiochemical estimation: Two millilitres of whole blood collected from the jugular vein of sheep and goats into EDTA coated vacutainers were used to analyse haematological parameters using BC-2800 Vet, Auto Haematology Analyzer, Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, China. The haematological parameters studied were total erythrocyte count (TEC) ($\times 10^6/\mu L$), haemoglobin (Hb) (g/dL), packed cell volume (PCV) (%), platelets ($\times 10^3/\mu L$) and total leucocyte count (TLC) ($\times 10^3/\mu L$).

About 4 ml of whole blood was collected using a serum vacutainer. Serum biochemical parameters, including total protein, albumin, globulin, albumin-globulin ratio, bilirubin, alanine transaminase, aspartate aminotransferase, gamma-glutamate transferase, creatinine, and blood urea nitrogen (BUN), were assessed. The analysis was performed utilizing the semi-automatic biochemistry analyser RX-50 (Microlab) and reagents manufactured by Transasia Bio-Medicals Ltd., Solan, HP.

DNA extraction and PCR: DNA extraction from the whole blood sample was carried out using the QIAamp DNA Mini Kit from Qiagen, Germany, following the manufacturer's instructions. Subsequently, a PCR assay was conducted for the amplification of specific genes. Confirmation was sought by performing PCR assays using genus-specific primers targeting the 18S rRNA gene of *Theileria* spp. and *Babesia* spp., as outlined by Allsopp *et al.* (1993) [2] and Duarte *et al.* (2011) [5] respectively.

Statistical analysis: Statistical analysis was conducted using GraphPad Prism software. The average values of haematological and biochemical parameters in the affected animals were compared with the average values of apparently healthy animals using an unpaired t-test. The results were expressed as Mean±SE, and statistical significance was set at $p<0.05$.

Results

The predominant clinical signs observed in haemoprotozoan infected animals were anorexia/inappetence, weakness, fever, pale conjunctival mucous membrane (Fig. 1), tachycardia, respiratory distress, diminished rumeno-reticular motility, lymph node enlargement (Fig. 2), haemoglobinuria, and a history of tick infestation (Fig. 3).

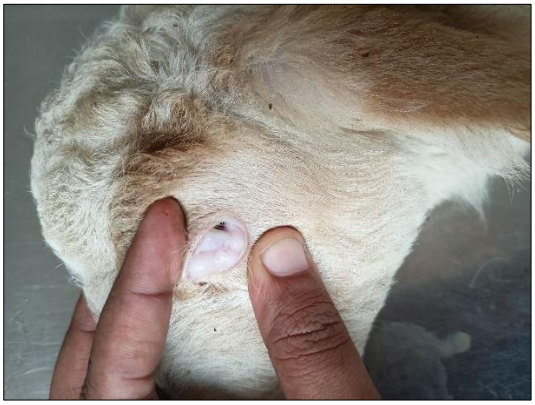


Fig 1: Pale conjunctival mucus membrane

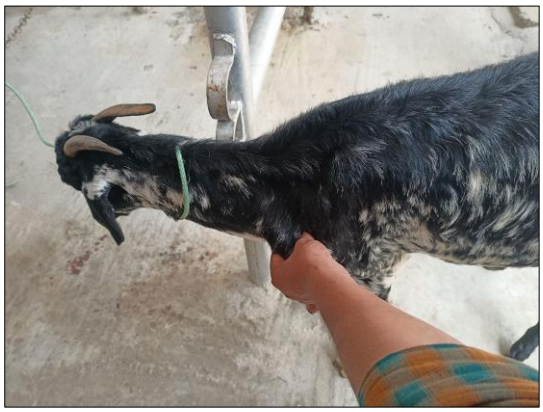


Fig 2: Enlarged lymph node

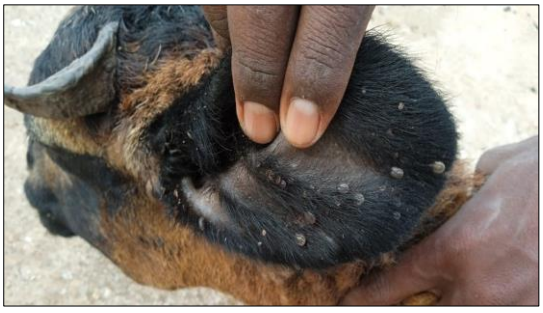


Fig 2: Tick infestation in haemoprotozoa affected animal

Upon examination of Giemsa-stained blood smears, it was observed that 6 animals were positive for *Theileria*, while 4 were positive for *Babesia* (Fig. 4a and Fig. 4b). Subsequently, DNA extracted from all infected animals underwent genus-specific PCR for molecular identification. The 989F and 990R

primer pair in the genus-specific PCR produced a 1098bp fragment (Fig. 5a), indicative of *Theileria* spp. Furthermore, the Bab7 and Bab 9 primer pair generated a 490 bp fragment (Fig. 5b), specific for *Babesia* spp. In the PCR analysis, 4 animals were found positive for *Theileria*, 4 for Babesia, and 2 for both *Theileria* and *Babesia* organisms.

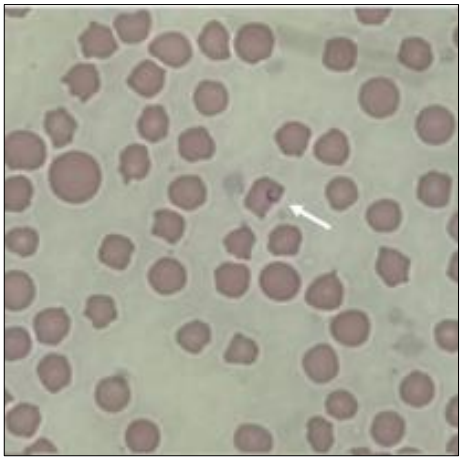


Fig 4a: Piroplasm of *Babesia* Spp. Giemsa 100X

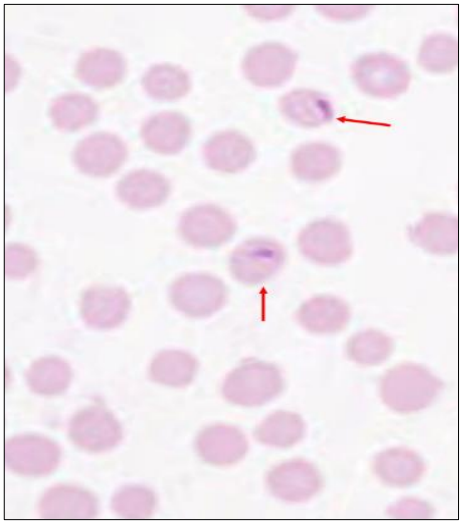
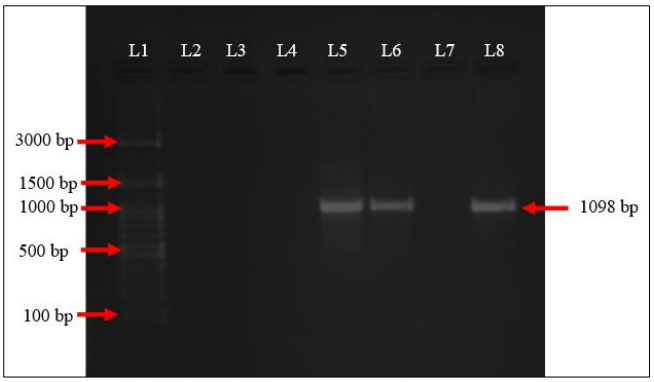
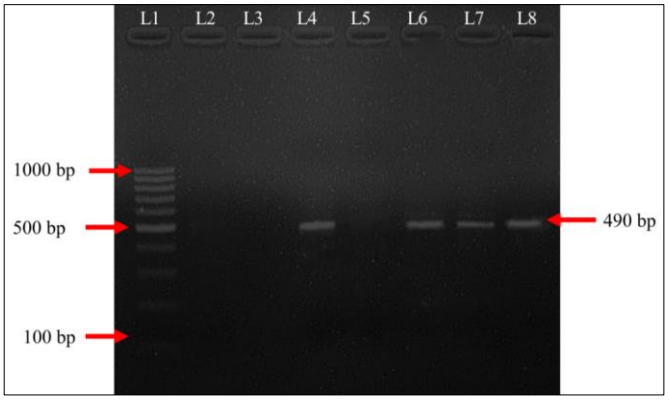


Fig 4b: Piroplasm of *Theileria* Spp. Giemsa 100X



(L1 – 100bp molecular DNA ladder, L5, L6 - Positive for *Theileria* genus, L8 - Positive control, L2 – No template control (NTC), L3, L4, L7 – Negative sample)

Fig 5a: The analysis of the amplified product (1098 bp) from blood samples was performed using the 989F/990R primer set targeting the *Theileria* genus.



(L1 – 100bp molecular DNA ladder, L4, L6, L7 - Positive for *Babesia* genus, L8 - Positive control, L2 – No template control (NTC), L3, L5 – Negative sample)

Fig 5b: The analysis of the amplified product (490 bp) from blood samples was performed using the Bab7/Bab9 primer set targeting the *Babesia* genus.

In the present study, the mean±SE of rectal temperature was 102.6±0.19°F and 104.2±0.47°F, respiration rate was 27.33±1.45/min and 32.10±1.34/min, heart rate was 80.33±0.42 beats/min and 88.30±1.30 beats/min, and rumeno-reticular motility was 3.83±0.31/3 min and 1.90±0.180/3min in healthy and haemoprotozoan-affected animals, respectively (Table 1). The observed physiological parameters, including temperature, respiratory rate, and heart rate, exhibited a statistically significant increase in animals infected with haemoprotozoa compared to the control group, while a statistically significant decrease in rumeno-reticular motility was noted in the affected group compared to the control group.

Table 1: The mean± SE values physiological parameters in healthy and haemoprotozoan affected small ruminants.

Sl. No	Parameter	Group I Healthy animal (N=6)	Group II Haemoprotozoa affected animals (N=10)	P Value
1	Temperature (°F)	102.6±0.19	104.2±0.47	0.03*
2	Heart rate (beats/min)	80.33±0.42	88.30±1.30	<0.01**
3	Respiration rate (/min)	27.33±1.45	32.10±1.34	0.04*
4	Rumeno-reticular motility (Per 3 minute)	3.83±0.31	1.90±0.180	< 0.01**

NS: Non-Significant at $p>0.05$ level; * Significant at $p\leq0.05$ level; ** Significant at $p\leq0.01$ level

In haematology, the mean± SE of haemoglobin was 12.95±0.32 g/dL and 6.63±0.75 g/dL, total erythrocyte count was 12.01±0.47 × 10^6/µL and 8.832±0.99 × 10^6/µL, total leucocyte count was 11.27±0.70 × 10^3/µL and 19.83±2.98 × 10^3/µL, platelets were 705.0±38.51 × 10^3/µL and 403.8±86.68 × 10^3/µL, and packed cell volume was 38.27±1.01% and 18.13±1.90% in healthy and haemoprotozoan-affected animals, respectively (Table 2). There was a statistically significant reduction in haemoglobin, total erythrocyte count, packed cell volume (PCV), and platelets in infected animals compared with the healthy control group, while the total leukocyte count exhibited a statistically significant increase in infected animals compared with the healthy control group.

Table 2: The mean± SE values haematological parameters in healthy and haemoprotozoan affected small ruminants.

Sl. No	Parameter	Group I Healthy animal (N=6)	Group II Haemoprotozoa affected animals (N=10)	P Value
1	Haemoglobin (g/dL)	12.95±0.32	6.63±0.75	< 0.01**
2	TEC (× 10^6/µL)	12.01±0.47	8.832±0.99	0.03*
3	TLC (×10^3/µL)	11.27±0.70	19.83±2.98	0.05*
4	Platelets (× 10^3/µL)	705.0±38.51	403.8±86.68	0.045*
5	PCV (%)	38.27±1.01	18.13±1.90	< 0.01**

NS: Non-Significant at p>0.05 level; * Significant at p≤0.05 level; ** Significant at p≤0.01 level

In serum biochemical estimation, the mean±SE of total protein was 6.88±0.07 g/dL and 6.33±0.11 g/dL, albumin was 3.00±0.07 g/dL and 2.46±0.07 g/dL, globulin was 2.03±0.05 g/dL and 1.87±0.04 g/dL, and albumin-globulin ratio was 1.48±0.04 and 1.30±0.03 in healthy and haemoprotozoan-affected animals, respectively (Table 3). There was a significant decrease in total protein, albumin, globulin, and albumin-globulin ratio in haemoprotozoan-affected animals compared with the healthy control group.

The mean±SE of total bilirubin was 0.52±0.03 mg/dL and 1.11±0.06 mg/dL, ALT was 330.17±0.65 IU/L and 50.93±1.01 IU/L, AST was 88.40±1.74 IU/L and 170.6±3.35 IU/L, GGT was 20.77±0.69 IU/L and 52.91±1.73 IU/L, serum creatinine was 1.27±0.04 mg/dL and 1.84±0.06 mg/dL, and BUN was 22.37±1.19 mg/dL and 29.77±1.30 mg/dL in healthy and haemoprotozoan-affected animals, respectively. A statistically significant increase in ALT, AST, GGT, total bilirubin, creatinine, and blood urea nitrogen was observed in haemoprotozoan-affected animals compared with the healthy control group.

Table 3: The mean± SE values serum biochemical parameters in healthy and haemoprotozoan affected small ruminants.

Sl. No	Serum biochemical Parameter	Group I Healthy animal (N=6)	Group II Haemoprotozoa affected animals (N=10)	P Value
1	Total Protein (g/dL)	6.88±0.07	6.33±0.11	0.01**
2	Albumin (g/dL)	3.00±0.07	2.46±0.07	<0.01**
3	Globulin (g/dL)	2.03±0.05	1.87±0.04	0.02*
4	Albumin globulin ratio	1.48±0.04	1.30±0.03	<0.01**
5	Total bilirubin (mg/dL)	0.52±0.03	1.11±0.06	<0.01**
6	Alanine transaminase (IU/L)	30.17±0.65	50.93±1.01	<0.01**
7	Aspartate aminotransferase (IU/L)	88.40±1.74	170.6±3.35	<0.01**
8	Gamma glutamate transferase (IU/L)	20.77±0.69	52.91±1.73	<0.01**
9	Creatinine (mg/dL)	1.27±0.04	1.84±0.06	<0.01**
10	Blood urea nitrogen (mg/dL)	22.37±1.19	29.77±1.30	<0.01**

NS: Non-Significant at p>0.05 level; * Significant at p≤0.05 level; ** Significant at p≤0.01 level

Discussion

Vector-borne protozoan diseases, *Babesia* and *Theileria* infections in small ruminants are recognized for causing significant health implications in affected animals (Onyiche *et al*., 2019) [17]. Polymerase chain reaction (PCR) is the most commonly used molecular technique for diagnosis of piroplasms compared to conventional blood smear examination (Gunes *et al*., 2016) [8]. In the present study, haemoprotozoan-infected animals exhibited prominent clinical signs such as anorexia/inappetence, weakness, fever, pale conjunctival mucous membrane, tachycardia, respiratory distress, diminished rumeno-reticular motility, lymph node enlargement, haemoglobinuria, and a history of tick infestation. These findings align with similar observations reported by Razmi *et al.* (2002) [20], Gunes *et al.* (2016) [8], Maharana *et al.* (2016) [15], Mahmoud *et al.* (2019) [16], and Stuen (2020) [24]. Cytokines, crucial for mediating and regulating the immune response to infection, play a pivotal role in inducing systemic inflammation. In parasitic infections, cytokines trigger a systemic inflammatory response, a significant aspect of haemoprotozoan disease pathophysiology, contributing to diverse clinical manifestations such as fever, anorexia, depression, pale mucous membranes, lethargy, enlarged lymph nodes, and splenomegaly (Karasova *et al*., 2022) [12].

In the present study, a significant increase in body temperature, respiratory rate, and heart rate was observed compared to the healthy group. The elevation in respiration rate and heart rate is interpreted as a compensatory mechanism aimed at maintaining the balance of oxygenated blood in response to the observed decreases in red blood cell (RBC) levels, hemoglobin (Hb), and packed cell volume (PCV) in haemoprotozoa-infected animals. These findings align with numerous previous studies, as reported by Constable *et al.* (2017) [4] and Mahmoud *et al.* (2019) [16]. There was a significant decrease in rumen contractions in haemoprotozoa-affected animals may be attributed to decreased muscle tone, lower serum calcium levels, and the release of histamines, potentially contributing to ruminal atony (Haq *et al*., 2021) [9].

In haematology, a statistically significant decrease in haemoglobin, total erythrocyte count (TEC), platelets, and

packed cell volume (PCV) was observed in haemoprotozoan-affected animals compared to healthy animals. Similar findings of reductions in haemoglobin, TEC, thrombocytes, and PCV have been reported in studies by Izzo *et al*. (2010) [11], Mahmoud *et al.* (2019) [16], Razmi *et al.* (2019) [21], Stuen (2020) [24], Haq *et al.* (2021) [9] and Sivajothi *et al.* (2022) [22]. The observed anaemia can be attributed to the destruction of red blood cells containing piroplasms, complemented by other contributing factors such as autoimmune haemolysis and a suboptimal response from the bone marrow (Constable *et al*., 2017) [4]. Additionally, the decrease in platelet count could be attributed to reduced marrow production, hypersplenism, and platelet consumption due to widespread endothelial damage in tick-borne haemoprotozoan infection (Pantanowitz, 2002) [18].

In haemoprotozoa-affected animals, a significant increase in leukocyte count, known as leukocytosis, was observed. This phenomenon can be attributed to the proliferation of lymphocytes in the lymphoid organs, reflecting a defensive response to haemoprotozoa infection (Razmi *et al*., 2019 and Sivajothi *et al*., 2022) [21, 22]. In contrast to the current findings of increased leukocytes, other studies by Mahmoud *et al.* (2019) [16] and Eliwa *et al.* (2021) [7] reported a significant decrease in leukocyte count, which might be attributed to the destruction of white blood cells (WBC), particularly lymphocytes, during the progressive stage of the disease.

In serum biochemical estimation, a statistically significant decrease in total protein, albumin, globulin, and albumin-globulin ratio was observed in haemoprotozoan-affected animals compared with the healthy control group, consistent with findings reported by Gunes *et al.* (2016) [8], Mahmoud *et al.* (2019) [16], and Eliwa *et al.* (2021) [7]. Hypoproteinemia and hypoalbuminemia in haemoprotozoa infection may result from multiple factors, including reduced synthesis in the liver due to liver failure, the release of proteinaceous fluids from diseased lymph nodes into the extravascular space, and a decrease in protein intake due to anorexia during the infection (Mahmoud *et al*., 2019) [16]. In contrast to the present finding of decreased serum protein, Izzo *et al.* (2010) [11] noted a significant increase in the levels of serum total protein and globulin in animals affected by theileriosis. This elevation in values could potentially be attributed to a chronic immune response in the affected animals. The reduction in the albumin-to-globulin ratio corresponds to an increase in inflammation leading to heightened production of acute-phase proteins caused by haemoprotozoan organisms (Agina *et al*., 2021) [1]. A slight elevation in globulin level and severe hypoalbuminemia led to a decrease in the albumin-to-globulin ratio.

There was significant increase in total bilirubin in infected animals the increased bilirubin levels can stem from both intravascular and extravascular destruction of parasitized erythrocytes, particularly through erythrophagocytosis in the spleen, lymph nodes, and other organs of the reticuloendothelial system. Additionally, factors such as reduced hepatic uptake of bilirubin, attributed to hepatic damage, and diminished biliary excretion, along with haemolytic anaemia, can contribute to this elevation (Pugh and Baird, 2012) [19].

In the present study, there was a significant increase in serum ALT, AST, and GGT levels in haemoprotozoan-affected animals, consistent with findings reported by Pugh and Baird (2012) [19], Mahmoud *et al.* (2019) [16], Eliwa *et al.* (2021) [7], and Haq *et al.* (2021) [9]. The presence of parasites in any tissue results in tissue damage. In cases of hepatic injury associated with haemoprotozoa, increased serum levels of AST, ALT, and GGT were closely linked to hepatic function. Moreover, the notable elevations in serum AST, ALT, and GGT activities were primarily attributed to muscle trauma caused by prolonged recumbency in haemoprotozoa infection (Hasanpour *et al*., 2008). The significant elevation in serum creatinine and blood urea nitrogen levels might be attributed to kidney damage (Col and Uslu, 2007) and increased protein catabolism due to anorexia during haemoprotozoa infection (Sivajothi *et al*., 2022) [22].

Conclusion

In conclusion, this study highlights the economic implications of haemoprotozoan infections in small ruminants. It emphasizes the crucial role of early diagnosis through conventional blood smear examination and PCR techniques, providing insights into the clinicopathological features for effective management and a better understanding of disease pathogenesis.

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