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Hemostatic Disorders in Sheep Parasitized by Haemonchus contortus

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Abstract

Haemonchosis remains, to this day, the principal disease encountered in global sheep farming. It is characterized by the parasitemia of sheep with the hematophagous parasite *Haemonchus contortus*. The parasite causes lesions in the gastrointestinal tract of infected animals. These lesions, along with the secretion of anticoagulant factors and proteins, facilitate the parasite's feeding through the bleeding induced at these sites. The blood loss caused by the parasite leads to anemia and excessive loss of proteins such as albumin, resulting in clinical symptoms of weight loss, increased morbidity, and reduced production efficiency, which causes significant economic losses to the industry and affects the well-being of the affected animals. This study aims to demonstrate that animals infected with *Haemonchus contortus* develop hemostatic disorders. The results showed a statistically significant difference between experimentally parasitized sheep and parasite-free sheep. Tests conducted included Activated Coagulation Time ($p = 0.0001$), Activated Partial Thromboplastin Time ($p = 0.0045$), and Prothrombin Time ($p = 0.0325$), all showing statistical significance ($p < 0.05$) between the control and experimental groups when compared using ANOVA. Additionally, significant differences were observed in hematocrit values ($p < 0.0001$) and platelet counts ($p < 0.0001$).

INTRODUCTION

The parasite *Haemonchus contortus* is a hematophagous gastrointestinal parasite with the highest prevalence in tropical and subtropical regions (Abdo et al., 2017). It affects sheep of all ages, making it a concern across all age categories (Oliveira et al., 2017). It is considered one of the major sanitary issues in sheep farming, leading to significant economic losses for producers (Sczesny-Moraes et al., 2010).

The pathogenesis of ovine haemonchosis is related to the damage caused to the abomasum by the parasite, which uses an oral lancet to draw blood from the mucosal vessels of the abomasum (González-Garduño et al., 2014), in addition to causing digestive disturbances (Hoste et al., 2016). Consequently, affected animals may exhibit weight loss, hypoproteinemia, and severe anemia (Alam et al., 2020).

Hemostasis is a physiological process aimed at maintaining blood in a fluid state within the blood vessels, preventing both hemorrhage and thrombosis (Thrall & Alisson 2015). The components involved in hemostasis include platelets, endothelial cells, blood vessels, plasma coagulation factors, physiological inhibitors of coagulation, and the fibrinolysis system [8]. These factors are regulated to ensure a balanced opposition between the control of excessive blood loss and the prevention of intravascular thrombus formation, which is associated with excessive fibrin formation (Thrall & Alisson 2015).

Given that hemorrhagic lesions and blood consumption are closely related to the pathogenesis of *Haemonchus contortus* parasitism, this study aimed to elucidate whether the parasite can induce alterations in the host’s coagulation process through the analysis of the intrinsic, extrinsic, and common pathways of coagulation (Hashimotto, 2006).

MATERIAL AND METHODS

Nineteen Texel-cross sheep, aged between 7 and 8 months and of both sexes, were used in the study. The animals were fed a diet consisting of 3% of their live weight in dry matter, which included roughage (silage) and concentrate (balanced feed specifically formulated for sheep), with water provided ad libitum.

The sheep underwent a 14-day adaptation period before the start of the experimental phase, which lasted 4 months. During this time, the animals were housed in two pens with slatted flooring, divided into two distinct groups:

- **G1: Control Group**, consisting of 5 sheep, confined for 4 months and subjected to fecal examination using the Egg Count Per Gram (OPG) technique, which was negative for strongyle eggs.
- **G2: Experimental Group**, comprising 14 sheep, also confined for 4 months, experimentally infected with *Haemonchus contortus*, and testing positive for strongyle eggs in the OPG.

Before the experiment began, all selected animals were treated with a broad-spectrum anthelmintic (Monepantel 2.5%) to eliminate any existing parasitic load. Following a negative fecal test for strongyle eggs using the OPG technique and the adaptation period, the infection protocol was initiated. Infective third-stage larvae (L3) used for the infection were obtained via coproculture (Roberts & O'Sullivan, 1950). The fecal donors were lambs with a *Haemonchus contortus* (Ueno & Gonçalves, 1998). For the experimental infection, a dose of 10,000 infective larvae was administered orally to each animal, divided into two applications of 5,000 larvae each, with the first application on day 0 of the experiment and the second application after an interval of 25 days.

Four blood collections were performed at 30-day intervals. Following hair removal and site antisepsis with 70% alcohol, blood samples were collected via puncture of the external jugular vein using a 0.70 x 25 mm needle and 10 mL disposable syringes. Blood collected in tubes containing 7.2 mg of K2 EDTA as an anticoagulant was used for hematocrit determination and platelet count. Blood collected in tubes without anticoagulant and with a clot activator gel was used for serum biochemical analysis, while blood collected in citrate tubes was used for plasma preparation for subsequent hemostatic profile analysis.

All procedures involving the animals were approved by the Ethics Committee on Animal Use under protocol CEUA Nº 7843220419 (CEUA-IFFar).

For hematocrit determination, blood from the K2 EDTA tube was placed in microcapillaries and centrifuged for 3 minutes at 3000 rpm. The hematocrit percentage was determined using a standardized table for the technique. Platelet counts were performed using an automatic hematological analyzer (Maxcel 200 Hematology Counter), with results manually corrected on blood smear slides.

Biochemical and Hemostatic Tests: Citrate tubes and tubes with clot activator gel were centrifuged for 5 minutes at 2500 rpm to facilitate clot formation or sedimentation of blood components. The serum was

pipetted into Eppendorf® microtubes, and the samples were stored in a freezer at -20ºC until the serum activity of Aspartate Aminotransferase (AST) and serum concentrations of Albumin (ALB) and Total Protein (TP) were determined using an automatic analyzer (Cobas® C111 Analyzer, Roche® Diagnostics Ltda, USA) with Roche® Diagnostics Ltda. (USA) kits. The plasma obtained was used after centrifugation for the tests of Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT), conducted on a semi-automatic biochemical analyzer using commercial kits (Gold Analisa® Diagnóstica Ltda, Carlos Prates, Belo Horizonte, Minas Gerais, Brazil).

For the Lee-White Activated Coagulation Time test, a stopwatch was started immediately after blood collection. Three milliliters of blood were distributed into three pre-warmed test tubes (37 ºC), with 1 mL allocated to each tube. The tubes were placed back into the water bath. Coagulation in the first tube was checked every 30 seconds. Once a clot formed in the first tube, the second tube was checked, and so on. The time was recorded after complete clot formation in the third tube, which was considered the blood coagulation time.

Determination of Eggs Per Gram of Feces (EPG): Fecal samples were collected biweekly directly from the rectal ampulla to determine the EPG using the McMaster technique (Gordon & Whitlock, 1939) - modified for monitoring the parasitological status of both the control and experimental groups.

On the first and last days of the experiment, the sheep from both the Control and Experimental groups were weighed individually. Additionally, weekly assessments of the ocular mucosa color of the animals in both groups were conducted.

Statistical analysis of the results was performed using ANOVA to compare pre- and post-infection changes between the experimental and control groups. A p-value < 0.05 was considered statistically significant.

RESULTS

The results obtained from tests related to blood hemostasis, including Activated Clotting Time ($p = 0.0001$), Activated Partial Thromboplastin Time ($p = 0.0045$), and Prothrombin Activity Time ($p = 0.0325$), showed statistically significant differences ($p < 0.05$) between the control and experimental groups when analyzed using the ANOVA test. Additionally, significant differences were observed in hematocrit levels ($p < 0.0001$) and platelet count ($p < 0.0001$).

Biochemical tests used to assess potential liver damage and organ function included serum aspartate aminotransferase (AST) activity ($p = 0.9098$), and serum concentrations of albumin ($p = 1.0000$) and total protein ($p = 1.0000$). These tests did not show statistically significant differences between the groups, indicating that the liver function of the animals in the experimental group was preserved, ensuring that the coagulation factors necessary for blood hemostasis were being synthesized.

Both Group 1 (G1) and Group 2 (G2) showed statistically significant differences in weight gain over the course of the experiment, based on the mean difference between initial and final weights over the 60-day period. G2 had an average weight gain of 6.335 kg ($p < 0.0001$), while G1 showed an average gain of 9.250 kg ($p = 0.0025$).

The animals in G1 remained negative for egg per gram (EPG) counts throughout the experimental period, whereas G2 exhibited a steady increase in EPG starting from the third month of the experiment, as shown in Fig. 1.

DISCUSSION

The liver is a crucial organ for hemostasis, as it is responsible for producing the majority of coagulation factors within hepatocytes (Torres et al., 2016). Although laboratory tests did not indicate any liver dysfunction or damage, the animals in Group 2 (G2) exhibited deficiencies in hemostasis, as evidenced by increased Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), and Activated Clotting Time (ACT). This can be explained by the fact that the parasitic load inoculated was not high enough to cause liver damage, as demonstrated by (Hupp et al., 2018).

Hemostasis is a physiological process aimed at preventing hemorrhage or thrombosis (Rezende 2010). Primary hemostasis involves the interaction between damaged vascular endothelium and platelets, leading to the formation of a platelet plug. Secondary hemostasis involves a series of coagulation cascade reactions, ultimately producing fibrin, which stabilizes the platelet plug (Ferreira et al., 2010).

A significant decrease in platelet counts was observed in parasitized animals, indicating platelet consumption related to primary hemostasis, which is necessary to stabilize bleeding caused by the parasite in the abomasum. Thrombocytopenia in animals parasitized by *Haemonchus contortus* has been reported in other studies (Cériac et al., 2017; Lima et al., 2022).

There is a reduction in serum ADP levels in sheep experimentally infected with *Haemonchus contortus*, and this decrease is correlated with impaired platelet aggregation and the formation of a hemostatic plug, which facilitates the parasite's feeding process (Gressler et al., 2014). These alterations hinder blood hemostasis, as effective hemostasis requires both an adequate quantity and quality of platelets (Morowski et al., 2013).

The Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT) tests are screening assays used to evaluate blood hemostasis. The APTT test assesses the intrinsic and common coagulation pathways, including factors VIII, IX, XI, and XII, as well as prekallikrein and high-molecular-weight kininogen. On the other hand, the PT test evaluates the extrinsic and common coagulation pathways, which involve factors VII, V, II, and X (Rizzati & Franco, 2001).

It has been identified that the parasite *Haemonchus contortus* can secrete a protein called calreticulin, which is capable of binding to calcium ions ($Ca2+$) and coagulation proteins such as factor X, thereby

acting as an anticoagulant and facilitating the parasite's ability to feed on the host's blood (Qamar et al., 2011).

Additionally, the *H. contortus* protein Hc-SPI-I8A functions as an inhibitor of blood coagulation through its TIL domain, hindering extrinsic blood coagulation by interacting with OaTSP1CP (a Thrombospondin Type 1 Protein Containing Domain from *Ovis aries*) through the same domain (Gressler et al., 2014). Furthermore, the parasite may also interfere with the intrinsic pathway of the coagulation cascade through unknown mechanisms. *H. contortus* may exert an anti-hemostatic effect via its serpina protein (Yi et al., 2010).

The data observed in this study suggest a significant interaction between the parasite and the host, where *H. contortus*, along with its proteins, has the ability to interfere with the coagulation cascade, thereby altering the hemostatic process. This interaction is evident through the prolonged Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), and Clotting Time (CT).

In this study, alterations in prolonged coagulation time were demonstrated through the tests for Activated Clotting Time ($p = 0.0001$), Activated Partial Thromboplastin Time ($p = 0.0045$), and Prothrombin Time ($p = 0.0325$). These findings are consistent with the secretion of Hc-SPI-I8A (Gressler et al., 2014) and serpina (Qamar et al., 2011) proteins, which act as anticoagulants, leading to increased coagulation times and inhibition of the hemostatic process.

On the other hand, hematocrit levels showed a statistically significant difference when comparing the control and experimental groups. However, this parameter remained within the reference range for the species (24% to 50%) throughout the experimental period, except for one individual that exhibited a hematocrit level of 21% and an egg per gram (EPG) count of 3400 in the fourth month of the experiment. It has been demonstrated that in experimental infections with 10,000 *Haemonchus contortus* larvae and an EPG count up to 2500, anemia does not typically manifest in sheep (Hupp et al., 2018). Therefore, the maintenance of hematocrit levels within the normal range can likely be attributed to the relatively low level of parasitic infection in the animals.

Weight loss in animals parasitized by *H. contortus* has a detrimental impact on production systems, leading to significant economic losses for producers (Qamar et al., 2011). When analyzing zootechnical indices, a reduction in weight gain of approximately 31.5% was observed in the experimental group compared to the control group. This reduction in weight gain is likely related to the parasitic behavior of *H. contortus*, as the lesions caused in the abomasum not only lead to blood loss but also result in digestive alterations and reduced nutrient absorption (Hoste et al., 2016).

Although there was a statistically significant difference in weight between the groups, it is evident that the control group achieved greater weight gain than the experimental group over the same period. Additionally, the ocular mucosa color of the animals was assessed weekly, and it was found to be normochromic in both groups throughout the experiment.

CONCLUSION

In conclusion, the results of this study demonstrate that the parasite *Haemonchus contortus* affects hematocrit levels, platelet counts, and impairs the ability to achieve adequate blood hemostasis in infected lambs.

Declarations

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CONFLICT OF INTEREST

The authors declare they have no competing interests.

DATA AVAILABILITY

The datasets generated during and analyzed during the current study are not publicly available due to they are needed for further research but are available from the corresponding author on reasonable request.

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Figures

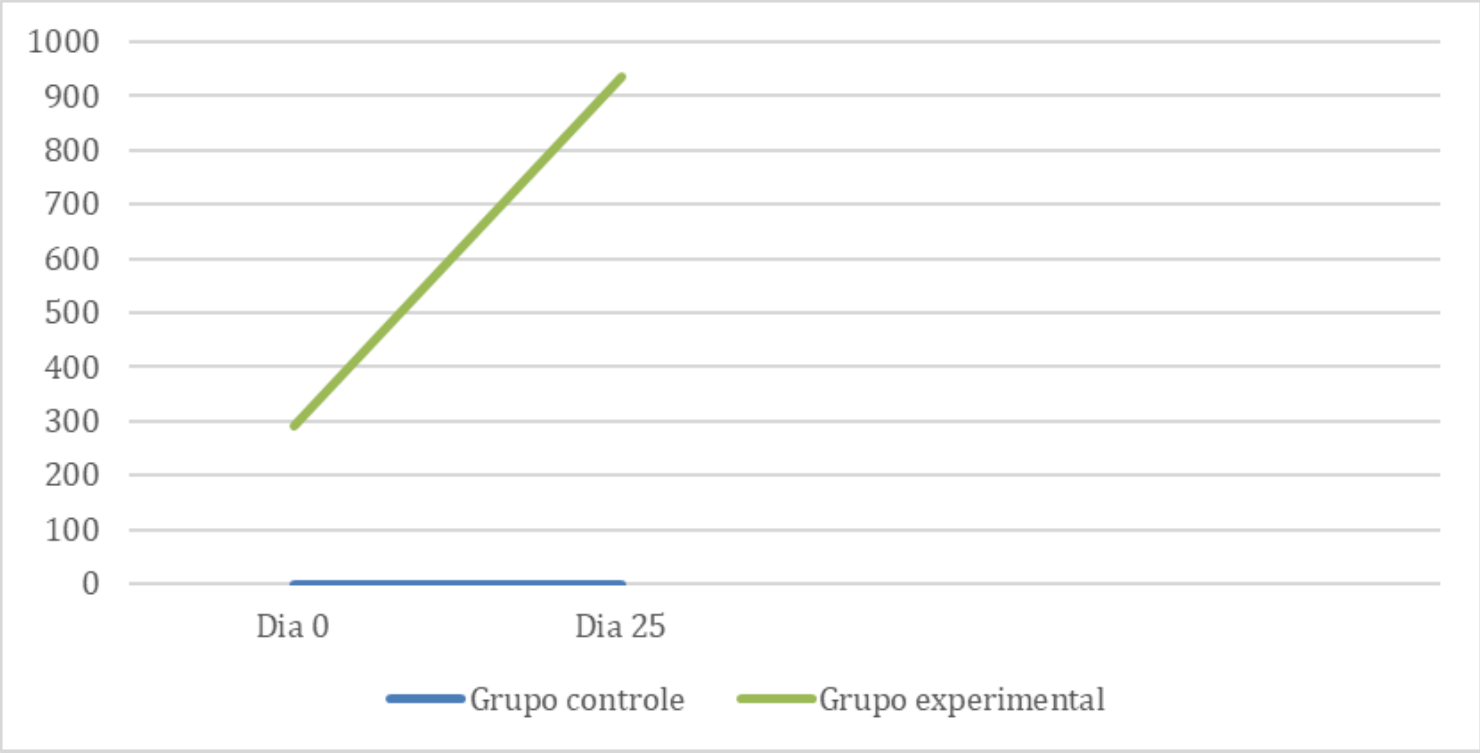


Figure 1

Monthly average egg counts per gram of feces in the control and experimental groups.