Host-parasite of Sheep Mucus Layers infected with *Dictyocaulus filarial*

Roberto Montes-de-Oca-Jiménez*, Betsy Coyote-Camacho, María-Eugenia López-Arellano, Cesar Ortega-Santana, Pomposo Fernández-Rosas, Humberto G. Monroy-Salazar, Esvieta Borroto-Tenorio

[1] Center for Research and Advanced Studies in Animal Health, Faculty of Veterinary Medicines and Animal Husbandry, Autonomous University of Mexico State (UAEM), Km 15.5 Autopista de cuota Toluca – Atlacomulco; Toluca, Estado de México, 50200, México.
[2] National Center for Disciplinary Research in Veterinary Parasitology. National Institute for Forestry, Agriculture and Livestock.Cuernavaca-Cuautlaroad#8534, Jiutepec, Morelos, Mexico.C.P.62550
romojimenez@yahoo.com

Abstract: The immune system has the essential function of protecting the body against the damaging effects of pathogenic microbial agents. The incidence of parasitic diseases, including respiratory helminthosis varies greatly from place to place depending on the relative importance of the factors. Several report the periods of high risk of infection by small lungworms. This study was performed in eight sheep farms with 112 sheep flocks located in Ixtapaluca Municipality, State of Mexico. Gross tissue examination was carried out considering localization, mucus layers as well as pulmonary consistency. Lesions were classified as: no lesion, mild lesion and severe lesion. In addition to the analysis of the infected tissue was used immunohistochemical techniques. Briefly, a set of primary monoclonal antibody (mAbs) was used to label macrophages, $CD4^+$, $CD8^+$, $\gamma\delta^+$ T. In this work, *D. filaria* infection was found in 47.6% of infected tissue, and 52.4% of pulmonary tissue damage showed the presence of *D. filarial,* nematode genera. The mucus layer, was the main tissues affected by lungworms, showing an increased number of immune cell population, compared with control group In addition, hosts with heavy lungworm infection showed increased number of $CD4^+$, $\gamma\delta^+$ T-cells and B^+ cells. The data provide further evidence that subsets of inflammatory cells into the lungs include sheep infected with lung worms, however, the understanding of the process of the immune response and the development of resistance in sheep lung worm remain to be clearly elucidated.
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1. Introduction

The incidence of parasitic diseases, including respiratory helminthosis varies greatly from place to place depending on the relative importance of the factors. Several report the periods of high risk of infection by small lungworms. In Ethiopia the periods of highest incidence were autumn, winter and early spring to late summer and early. On the other hand most studies focussing on nematode infestations in Germany have reported a moderate to high prevalence of infection (51% and 100%) in sheep (Moritz 2005). Addition in Norway Lowest *Dictyocaulus* prevalence and intensity were found in calves in June, presumably due to limited exposure prior to June sampling (calving occurs in early May) and the pre-patent period prior to larval excretion, but levels approached those of yearlings by August. Highest *Dictyocaulus* infection intensity was seen in yearlings. Lungworm prevalence increased with age, but infection intensity (LPG) tended to decrease with age. Adults had significantly lower *Dictyocaulus* larval burdens in August than June, suggesting either immunological development, as reported with *Dictyocaulus viviparous* (Hagberg 2008), or seasonal variation.

However, most adult worm infection and increased excretion of larvae was observed in late autumn and winter, when heavily infected snails. Helminth parasites of ruminants are ubiquitous, with many tropical and subtropical environments of the world providing nearly perfect conditions for their survival and development(Alemu, Leykun et al. 2006). *Dictyocaulidae* certain are known to exist in Mexico. (Manrique-Saide, Escobedo-Ortegón et al. 2010). On the other hand very few and limited studies were done so far pertaining to respiratory helminthes of small ruminants in the México. Several endoparasites, including *Dictyocaulus filaria*, are cause of death and morbidity in the Mexico State.

Infections with gastrointestinal nematodes can negatively affect the health and the overall productivity of infected animals (Suarez, Cristel et al. 2009). Therefore, they can be a major cause of economic losses in small ruminant production. The common clinical signs of an infection with gastrointestinal nematodes are anorexia, diarrhoea,

emaciation and anaemia (Behrens H 2001). Today, the control of gastrointestinal nematodes in sheep has become less effective due to the development of anthelmintic resistance (Fleming, Craig et al. 2006).

The capacity of helminth parasites to modulate the immune system underpins their longevity in the mammalian host (Hewitson, Grainger et al. 2009). The immune system has the essential function of protecting the body against the damaging effects of pathogenic parasitic agents. This system is activated by the processing and presentation of antigen by antigen presenting cells to T lymphocytes. Helminths are well adapted to their host and have evolved sophisticated strategies to avoid or modulate the host immune defense. Although the exact mechanisms via which helminthes subvert the host responses are not completely understood, they typically induce an anti-inflammatory environment (Maizels, Balic et al. 2004, Wilson and Maizels 2004).

Definitive diagnosis of degenerated lesions is often difficult using gross, stereoscopic or histological examination methods since the lesions no longer contain identifiable parasite features, and are consistent histologically with chronic lesions of other etiologies (Scandrett, Haines et al. 2012). Several techniques used for diagnosing nematode example immunohistochemical techniques. (Tawfeek, Elwakil et al. 2011) (Atienzar, Tilmant et al. 2011). Furthermore Molecular morphology provided a combination of morphological details of histological sections with simultaneous display of target antigens. The immunohistochemical technique is a powerful diagnostic tool (Haines and West 2005).

Materials and Methods

1.1. Biology assays

1.1.1. Area of Study and experimental animals

This study was performed in eight sheep farms with 112 sheep flocks located in Ixtapaluca Municipality, State of Mexico. Ixtapaluca is a small village located at 10°16´33´´N latitude, 2500 m above sea level, with a mean temperature of 15°C from January to December. Twenty- one native sheep from 12 to 18 months old were selected based on the presence of lungworm larvae in faecal samples by the Baermann technique. A group of 20 free-nematode sheep were chosen from Universidad Autónoma del Estado de México and were included as control group.

1.1.2. Lungworm larvae and adult identification.

Infected sheep with pulmonary nematodes were euthanized using an overdose of anaesthesia (according to NOM-033-ZOO-1995, which dictates humanitarian sacrifice of animals in Mexico) and necropsy was carried out. Adult nematodes were picked up from pulmonary tract and lung-larvae were collected following Henriksen's technique (1965). Ten g of lung tissue sections were cut and incubated at 30°C with distilled water in cloth sieves for 24 h. Then, larvae were centrifuged at 165 g at 4°C during 5 min and preserved with 10% formaldehyde until used. Taxonomic identification of lung nematodes was performed according to the morphometric keys described by Liébano-Hernández (2004).

1.1.3. Pathological techniques

Gross tissue examination was carried out considering localization, mucus layers as well as pulmonary consistency. Lesions were classified as: no lesion, mild lesion and severe lesion. Lung sections fixed in 10% formaldehyde were dehydrated in alcohol and paraffin embedded at 56°C using an automatic tissue processor (Histokinette 8000 Reichert-Jung, NY, USA). Five micrometre thick tissue sections were cut out and stained with Hematoxylin-Eosin (H.E.), Masson´s Trichromic and Toluidine blue to identify affected tissues, eosinophils and mast cells infiltration, respectively. In addition, the number of eosinophils and mast cells were obtained from 10 randomly selected fields using a 40x objective. Also, histopathological lesions were associated to the parasitic nematodes found in 10 tissue fields using 4x, 10x and 40x objectives.

1.1.4. Analysis of infected tissue by Immunohistochemistry

Small tissue samples were embedded in 2-methyl-butane at -196°C and kept at -70°C until used. Thick-tissues of 5 µm were cut using a Cryocut cryostat 1800 (Reichert Jung, Germany), transferred onto microscope slides covered with polylysine (Sigma, St. Louis, USA) and fixed with acetone for 1 min. Samples were kept at -20°C until used. Next for immunohistochemical assays an ImmunoDetector HRP/DAB commercial kit (Bioscience, Cal, USA) was used, following the manufacturer's instructions. Briefly, a set of primary monoclonal antibody (mAbs) was used to label macrophages, $CD4^+$, $CD8^+$, $\gamma\delta^+$ T and B^+ cells as shown in (**Table 1).** Fixed tissue sections were soaked in a peroxidase blocking solution for 5 min to reduce endogenous peroxidase reaction, and sections were washed after each step with 0.05 M tris-buffer solution (TBS). Tissue sections were incubated at 37°C for 1h and 15 min with the primary mAb and the secondary antibody (Biotinylated Anti-Mouse and Anti-Rabbit conjugated with horseradish peroxidase, HRP), respectively. Reaction was developed with 3,3´-diaminobenzidine tetrahydrochloride for 5 min and stopped with TBS. Mayer's hematoxylin was used as counter stain. Negative tissue controls were treated in the same way using TBS instead of the primary antibody. In addition, macrophages, B and T cells were counted in 10 different fields using a 40x objective in each animal sample.

Table 1. Surface monoclonal antibodies markers designed to recognized macrophages, $CD4^+$ T, $CD8^+$ T, $\gamma\delta^+$ T cells and lymphocyte B^+ on infected lung cells with parasitic nematodes.

mAb	Cell target	Specificity	Isotype	Dilution
VPM 65[3]	$CD14^+$	Macrophages	IgG_1	1:30
17D1[2]	$CD4^+$	T helper cells	IgG_1	1:400
CC63[3]	$CD8^+$	T cytotoxic cells	IgG_{2a}	1:100
HM3800[1]	T γ/δ^+	T gamma/delta (T γ/δ^+) cells	Anti-mouse TCR gamma delta	1:100
CC14[3]	CD1b	Lymphocytes B^+	IgG_1	1:100

1.1.5. Statistical Analysis of experimental assays

Statistical *t* student analysis was used to correlate the number of adult and larvae parasites with specific pathological lesions.In addition the Factorial Statistical Analysis Test (SSP® program, version 2.74, Gary Smith Corporation, USA) was used to evaluate the variation in leucocytes according to the number of parasites in lung tissue (Infante *et al*., 2005).

2. Results and Discussion

2.2. Experimental-Theoretic Study of *Dictyocaulus filarial*

2.2.1. Experimental results of gross and histologic analysis

In this work,*D. filaria* infection was found in 47.6% of infected tissue, and 52.4% of pulmonary tissue damage showed the presence of *D. filarial,* nematode genera.The diagnosis of parasitic infections in small ruminants has great importance due to the major threats this group of diseases pose to the welfare and productivity of ruminants and particularly of grazing sheep (Demeler, Schein et al. 2012). The percentages of identified lungworms genera were as followed: 71.4% and 52.4% for *D. filaria* larvae and adults.The histology examinations from infected tissue showed peribronchial and alveolar fibrosis adjacent to nematode sections with infiltration of lymphocytes, macrophages and alveolar mineralization. Multifocal alveolar emphysema was observed as well as interstitial widening caused by fibrosis and non-supurative inflammatory cells. On the other hand, a major morphological characteristic in our test was the larvae were obtained from infected sheep lung **(Figure 1).** *D. filaria* larva showing head the cephalic button.

In addition, different tissue damaged showed high quantity of eosinophils and mast cells compared to control group. Specifically, mucus layers tissue. The mean of infected sheep was around 8.3 ± 3.8 and 6.8 ± 3.2 to eosinophils and mast cells, respectively. In contrast, control group showed a media of eosinophils around 0.41 ± 0.1 and 0.38 ± 0.08 mast cells ($p<0.05$). There has been a considerable increase in eosinophils and mast cells. Chronic mast cell-mediated inflammation may contribute significantly towards the extensive tissue remodelling that is a feature of lungworm infection in ruminants (Collie, MacAldowie et al. 2001).

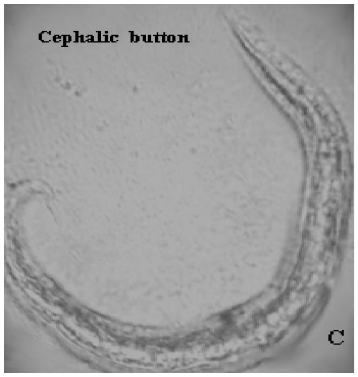

Figure 1. Main morphological characteristics of pulmonary larvae collected from infected sheep. C. *D. filaria* larva head showing the cephalic button.

2.2.2. Immunohistochemistry

Table 2 and Figure 2 come here.

Table 2. Macrophages surface markers detected in Mucus Layers of naturally infected sheep.

Tissue Cell markers	Mucus Layers	
	A	B
Macrophages	0.5 ± 0.0^a	4.0 ± 1.0^b
T $CD4^+$	1.7 ± 0.2^a	8.8 ± 0.6^b
T $CD8^+$	0.2 ± 0.05^a	4.1 ± 0.7^b
T γ/δ	1.4 ± 0.1^a	12.7 ± 0.9^b
B Lymphocytes	0.6 ± 0.1^a	14.6 ± 1.8^b

A= Control group; B= infected sheep group. $^{a,b}p < 0.01$

The mucus layer was the main tissues affected by lungworms, showing an increased number of immune cell population, compared with control group **(Table 2)**. In addition, hosts with heavy lungworm infection showed increased number of $CD4^+$, $\gamma\delta^+$ T-cells and B^+ cells. In recent reports were expressed numerous inflammatory cells including mast cells, eosinophils, T cells (Gulbahar, Davis et al. 2009). In this work naturally infected sheep with lungworms showed enhanced specific macrophages, $CD4^+$, $CD8^+$ and $\gamma\delta$ T-cells and B^+ lymphocyte response, compared with the control group ($p<0.05$) **(Figure 2).** On the other hand Kooyman in 2007 findings indicate that the IgG2/ IgG1 ratio (which may reflect the Th1/Th2 ratio) correlates with protection against *D. viviparus* infection (Kooyman, de Vries et al. 2007).

In comparison to other infectious agents such as protists and viruses, helminths are metazoans with significantly larger genomes that encode for complex programmes of development. On aspect of the increased developmental complexity of helminths is that, for a given species, different developmental outcomes may be possible at certain stages in the life

cycle. In the context of a helminth infection, developmental plasticity on the part of the pathogen may have important consequences for the host, determining the type and degree of pathology that develops, or whether pathology occurs at all (Davies and McKerrow 2003).

The data provide further evidence that subsets of inflammatory cells into the lungs include sheep infected with lung worms, however, the understanding of the process of the immune response and the development of resistance in sheep lung worm remain to be clearly elucidated. Coinciding with the results of our study.

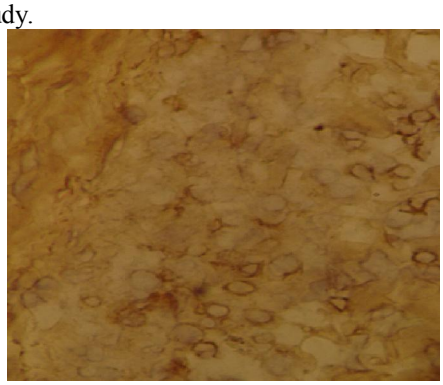

Figure 2. Immunohistochemical presence of T lymphocytes labeled with CD8

Appendix A. Supplementary data

Supplementary data to this article can be found online

Corresponding Author:
Dr. Roberto Montes de Oca Jimenez
Center for Research and Advanced Studies in Animal Health, Faculty of Veterinary Medicines and Animal Husbandry, Autonomous University of Mexico State (UAEM), Km 15.5 Autopista de cuota Toluca – Atlacomulco; Toluca, Estado de México, 50200, México
Email: romojimenez@yahoo.com

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