THE CLINICAL AND PATHOLOGICAL EFFECT OF *PROTOSTRONGYLUS STILESI* ON BIGHORN X MOUFLON HYBRID SHEEP[1]

by

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ABSTRACT

Blood parameters were measured in an attempt to find a clinical method of assessing lung damage and to correlate the level of *Protostrongylus stilesi* infection with clinical changes and lung damage. These parameters were: the complete blood count, blood pH, sedimentation rate, color index, mean corpuscular hemoglobin concentration, serum electrolytes, serum proteins, albumin/globulin ratio and the pattern of lactate dehydrogenase isoenzymes. A method to quantify physical lung damage was devised. The *P. stilesi* infection caused nodular formation in lung tissue, an indication of a slight increase in the proportion of eosinophils in peripheral blood and a slight increase in the serum bicarbonate concentration. No other alteration was noted. The conclusion was made that the level of *P. stilesi* parasitism achieved was not great enough to be detrimental to the experimental animals.

INTRODUCTION

Protostrongylus stilesi is a widespread parasite of bighorn sheep (*Ovis canadensis*). There is no reliable method to determine the extent of an infection of *P. stilesi* without sacrificing the animal. The method most widely used to estimate the intensity of a lungworm infection is that of Baermannizing weighed fecal samples and calculating the number of first stage larvae per gram of feces. Forrester and Senger (1964) found this method unacceptable because of extreme variation between fecal samples and even between individual fecal pellets from the same defecation.

The present study was undertaken to find a clinical method to indicate the extent of lung damage caused by lungworms (*P. stilesi*) in wild sheep. Blood parameters were selected, since blood is a relatively easily obtained substance and the quantity needed for the tests would not harm the animal.

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Protostrongylus-free bighorn sheep were not available. Therefore, *Protostrongylus*-free bighorn X mouflon hybrid sheep were used for this study. The assumption was made that the hybrids would become infected under the test conditions since both bighorn and mouflon sheep are susceptible to *P. stilesi* (Dikmans 1931, Howe 1965).

Blood was drawn from experimental animals before and during infection experiments. The following blood categories were examined: red blood cells, white blood cells, serum electrolytes, serum proteins and serum LDH isoenzymes. Estimation of physical damage to the lungs by the lungworms and possible histopathological effects to other body organs were also considered.

MATERIALS AND METHODS

The animals used for this study were Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) x mouflon sheep (*Ovis musimon*) hybrids. These animals were selected because of the difficulty in obtaining lungworm-free bighorns. A total of four sheep were studied. Sheep #1 was a 1/4 bighorn x 3/4 mouflon two-year-old ram. Sheep #2 was a 1/4 bighorn x 3/4 mouflon three year old ram. Sheep #3 was a second generation 1/2 bighorn and 1/2 mouflon one-year-old ewe. Sheep #4 was a second generation 1/2 bighorn x 1/2 mouflon two-year-old ram. Sheep #1 and #2 and sheep #3 and #4 were studied together.

The experimental animals were maintained in an isolation shed at Rachelwood Wildlife Research Preserve at New Florence, Pennsylvania. The windows of the shed were covered with double screening and the base of the building was circled by a Creosol-filled trough to protect the animals.

The infective stage larvae were administered to the sheep by feeding infected snails in gelatin capsules. Sheep #1 and #2 were each fed 345 infective stage larvae in 14 doses during the prepatent period. Sheep #3 was fed 203 infective stage larvae (11 doses) during the prepatent period and 173 infective stage larvae (7 doses) during the patent period. Sheep #4 was fed 162 infective stage larvae (9 doses) during the prepatent period and 210 infective stage larvae (9 doses) during the patent period. Fecal samples were collected at regular intervals in an attempt to monitor the infection intensity. Lungworm infections were established in three of four sheep (Monson 1971).

Three blood sampling periods were decided upon. The preinfection period constituted those blood samples drawn before infection was attempted. The prepatent period was initiated when infective stage *P. stilesi* larvae were being fed to the sheep. This lasted until the start of the patent period or that period when first stage larvae were being eliminated from the animals in the feces.

Blood was drawn from the jugular vein of the experimental animals with a California bleeding needle. The animals were caught by hand and held for bleeding. Approximately 25 ml of blood was taken each

time. Two tubes of blood were taken for blood serum requirements. One tube of blood was treated with Sequester Sol for blood tests requiring anticoagulated blood. Another tube of blood was heparinized and kept in an ice bath for pH determination. The tests requiring the use of whole anticoagulated blood were completed at the Rachelwood Wildlife Research Preserve directly after bleeding. Serum was prepared by allowing the blood to clot at room temperature, ringing the clot and centrifuging for 10 minutes. In some cases, the clot was removed prior to centrifuging. The serum was drawn off with a Pasteur pipette. The serum was then frozen and transported to Fort Collins, Colorado where the remainder of the tests were completed.

WHOLE BLOOD PARAMETERS

The packed cell volume (PCV) was determined by the micro hematocrit method. The red (RBC) and white (WBC) blood cell counts were determined microscopically. Hemoglobin (Hb) was determined by the cyanmethemoglobin method. The color index (CI) was calculated as follows:

$$CI = \frac{Hb(g/100ml)}{RBC \text{ in millions} \times 3}$$

The mean corpuscular hemoglobin concentration was calculated as follows:

$$MCHC = \frac{Hb\ (g/100ml) \times 100}{PCV}$$

The Wintrobe-Landsberg method was used to determine the erythrocyte sedimentation rate (ESR) except the time period examined was extended to 24 hours instead of the customary one hour. The differential leucocyte count was done on blood smears on standard microscope slides stained with Wright's stain. Blood pH was determined by means of an electronic pH meter.

SERUM BLOOD PARAMETERS

Serum bicarbonate was determined by the titration method. Sodium and potassium were determined by flame photometry. Total serum protein was determined by the biuret technique. Protein fractionization and lactate dehydrogenase (LDH) isoenzyme determination were done by electrophoresis of serum on cellulose acetate. The strips were scanned with a densitometer. Albumin, alpha globulin, beta globulin and gamma globulin percentages and the five LDH isoenzyme percentages were calculated from data determined by automatic integration.

DETERMINATION OF LUNG INVOLVEMENT

Linear slices were cut from anterior to posterior of the central part of the diaphragmatic lobes of the lungs of sheep #3 and #4. Histological sections of these lung slices were cut from parafin and stained with hematoxylin and eosin. First stage larvae were counted

microscopically throughout the linear slices of lung tissue. Areas of nodular and non-nodular involvement in the linear slices were calculated.

Lungworm nodules outside of the linear slices were removed from the lungs, counted and weighed. Adult worms were teased from the nodular tissue, counted, and identified (Monson 1971).

The remaining lung tissue was minced and Baermannized. The number of first stage larvae from the Baermannized lung and the larvae teased from the nodules were calculated to produce a total larval count exclusive of the tissue sectioned.

Histological sections of liver, spleen, kidney, myocardium, adrenal glands and spinal cord from sheep #3 and #4 were cut from parafin and stained with hematoxylin and eosin. These sections were examined for possible effects of lungworm involvement.

RESULTS

There were no significant differences in results obtained at the achieved level of parasitism for PCV, Hb, RBC, CI, MCHC, ESR, WBC, blood pH, serum sodium and potassium, total protein, protein fractions, the albumin:globulin ratio and lactate dehydrogenase isoenzymes. There was an indication of an increase in the serum bicarbonate and in the number of eosinophils. The other leucocytes remained proportionately normal. The hematological values possibly affected by *P. stilesi* are given in Table 1.

The relationship of the infection of *P. stilesi* to the damage found in the lung tissue is given in Table 2. Histopathologic results which were found in tissue sections cut from slices of lung tissue from sheep #3 and #4 revealed nodular areas. These areas contained adult lungworms, first stage larvae and eggs. The nodular area was small in comparison to the entire lung. They showed the usual symptoms of verminous pneumonia with lymphocytic infiltration, scarring and an occasional giant cell. Alveoli in these areas were non-functional. The lung tissue of both animals immediately outside the nodule and throughout the organ was normal for sedentary animals.

Wandering *P. stilesi* first stage larvae were extremely rare in the non-nodular lung tissue. Those that were present were found adjacent to the parasitic caused nodules.

The histology of the liver, kidney, spleen, adrenal gland, myocardium and lumbar spinal cord was considered to be normal in sheep #3 and sheep #4.

DISCUSSION

The bighorn sheep x mouflon sheep hybrids used for this study were very satisfactory. Trauma experienced during bleeding and other handling was minimal due to the animals being partially tame.

Table 1 - Hematological values possibly affected by *Protostrongylus stilesi*

	Bicarbonate meq/l			Eosinophils %		
	Mean	Range	SS	Mean	Range	SS
SHEEP #1						
Preinfection	23.0	21.3-24.7	2	1.3	1-1.7	2
Prepatent	21.8	17.1-28.5	9	4.7	1-10.5	10
Patent	22.7	16.5-30.7	4	6.0	4-8	4
SHEEP #2						
Preinfection	25.2	22.5-27.9	2	4.0	2-6	2
Prepatent	21.7	14.7-26.8	12	8.3	2-15	14
SHEEP #3						
Preinfection	17.3	11.5-25.1	6	1.2	0-3	6
Prepatent	19.7	13.3-24.8	10	5.8	2-12	10
Patent	22.1	17.6-29.4	8	4.0	1-9	8
SHEEP #4						
Preinfection	18.7	16.4-21.1	5	2.8	1-6	6
Prepatent	20.0	17.6-24.8	8	4.3	1-6	5
Patent	22.3	17.5-28.8	11	2.3	0-6	11

SS = sample size

Table 2 - Lung involvement with *P. stilesi* infection

	Sheep #3	Sheep #4
Infectious stage larvae fed	376	372
Number of nodules in lung tissue*	18	4
Adult worms recovered in lung tissue**	2	14
First stage larvae recovered from lung tissue	6,910	23,100
Area of lung slices scanned histologically (mm^2)	1,037	2,214
Area of lung tissue slices disrupted by lungworms (mm^2)	43	18
Percent of lung tissue slices disrupted	4.2	0.8
Weight of lung tissue (g)	224	381
Weight of nodular tissue (g)	6.0	3.6
Percent of nodular tissue	2.7	0.9
First stage larvae in lung tissue/g of lung tissue	31	60
Adult worms in histological slides of lung slices	4	3
First stage larvae in histological slides of lung slices found outside of formed nodules	5	4

Sheep #1 was fed 345 infective stage larvae. There were five nodules with six adults.

Sheep #2 was fed 345 infective stage larvae. No nodules were seen. No worms were recovered.

* Lung tissue refers to the tissue of the lung minus the slices removed for histological sections

**The number of adult worms are those with identifiable male and female parts. Other portions of worms were disregarded.

Three of the four sheep accepted the *P. stilesi* infection. This success was probably achieved because both bighorn sheep and mouflon sheep are susceptible to *P. stilesi* (Dikmans 1931, Howe 1965).

Effects of *P. stilesi* on the animals would be expected to be an immunological response by the animal and/or decreased lung function. The decreased lung function would occur only after the infective stage larvae reached the lung. Lung function would be most disrupted when the presence of the mature worms had resulted in nodular information and the production and movement of first stage larvae through the lung tissue.

Some of the blood parameters used in the present study would be more sensitive to the presence of the parasite rather than any disruption the parasite might cause. This would be expected with tests which would monitor immune reactions such as a change in the gamma globulin fraction.

The hematology of sheep #1 and sheep #2 was not tested consistently as the possibility of transmission of an infection of *P. stilesi* was not certain. Once a method of infection and procedures for testing blood parameters had been established, blood samples were drawn with more regularity from sheep #3 and sheep #4.

Sheep #2 did not become infected with *P. stilesi*. A prepatent method was established nonetheless. The animal was challenged by infective stage *P. stilesi* larvae and a reaction by the animal to this challenge was expected.

Sheep #3 became pregnant and aborted a half resorbed fetus 89 days into the parasitic patent period. This was accompanied by excessive vaginal exudate and inflamation and must be considered one of the factors causing changes in the hematological values for this animal.

The WBC differential in peripheral blood is the enumeration of the relative proportions (percentages) of the various types of WBC as seen in stained films (Lynch et al 1969). Leucocytosis is usually due to an increase of only one type of cell and is given the name of the increased cell type (Bauer et al 1968). Therefore, eosinophilia would be expected in increased parasitism based on past research.

No appreciable change in the leucocyte count was noted in the present study. All four sheep indicated a slight eosinophilia from the preinfection to the prepatent period. Sheep #1 continued the increase of eosinophils into the patent period. The percent of eosinophils decreased during the patent period for sheep #3 and #4 (Table 1). The rise for sheep #2 may have been part of a successful attempt to resist infection. The eosinophilic response in the experimental animals was similar to results reported by Poynter and Selway (1966), Weber (1957), and Wintrobe (1967). Trends in the change of neutrophils, lymphocytes and monocytes were inconclusive. The basophils during the patent period of sheep #3 may have been due to the abortion. Djafar et al (1960) found no alterations in the percentage of basophils or monocytes by increased parasitism.

Decreased pulmonary function results in an increase of H^+ in the blood. Changes in the concentration of H^+ in the extracellular fluid are accompanied by passage of a K^+ in the opposite direction. Serum K concentration is increased in acidosis purely as a result of changes in pH (Cantarow and Trumper 1962).

Sodium concentration of the blood serum is expected to remain normal with decreased pulmonary function. Serum bicarbonate is expected to increase as a result of renal compensation for acidosis. This would result in bringing the blood pH back to normal if a decrease occurred. Therefore pulmonary parasitism of a magnitude which would cause physiological or biochemical effects would decrease blood pH, increase serum bicarbonate, increase serum K and maintain a stable serum Na.

The blood pH did not decrease in any of the four experimental animals. Serum bicarbonate showed a slight progressive increase in sheep #3 and #4. There was no change in the bicarbonate of sheep #1. No rise was expected nor achieved from sheep #2 as there was no lung involvement. Serum K showed a decrease instead of the expected increase. Serum Na remained normal.

An explanation of these findings is that the destruction of pulmonary function was too insignificant or that tissue damage was gradual and the renal H^+ excretory and HCO_3- generative processes kept pace showing no marked increase in H^+. This is in agreement with Refsum (1966). There was no rise in K since the H^+ concentration did not increase. The K decreased as would be expected with a rise in pH. The rise in pH was explained by Refsum (1966) as a pathologic condition tending to cause metabolic alkalosis which sometimes leads to reduced H^+ concentration.

Many alveoli in the lungs of the experimental animals were not being utilized. The confinement of the sheep would greatly reduce their ability to exercise. This would be expected to cause a decrease in lung function.

The animals' defense mechanisms seem to have efficiently walled off the lungworm infection from the remainder of the lung tissue. This resulted in the formation of nodular elements and scar tissue. Also, there were few larvae outside the nodular area,and the larvae that were outside had not wandered far from the nodule. Lymphocytes and giant cells had invaded the area. The method used for estimation of lung damage in these animals could be used as an index for hunter-killed bighorn sheep as well as for collected specimens.

CONCLUSION

The level of lung involvement in the *P. stilesi* infection caused localized nodular verminous pneumonia, an indication of a possible increase in the proportion of eosinophils and a possible increase in the bicarbonate concentration. There were no other alterations in blood chemistry or hematology beyond the usual fluctuations. The

conclusion was made that the level of *P. stilesi* parasitism achieved was not great enough to be detrimental to any of the experimental animals.

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DISCUSSION

QUESTION BY CHARLES HIBLER, CSU: Sara, these sheep had three lungworms, right? Three species?

REPLY BY MCGLINCHY: They had one at necropsy.

REPLY BY HIBLER: You didn't find any *Muellerius* or *Pneumostrongylus*?

REPLY BY MCGLINCHY: No, we did not.

REPLY BY HIBLER: Did you find *Ostertagia* or *Trichuris*?

REPLY BY MCGLINCHY: There were a few adult *Ostertagia* recovered.

REPLY BY HIBLER: What is one of the cardinal signs of parasitism?

REPLY BY MCGLINCHY: Eosinophilia.

REPLY BY HIBLER: Anyway, I understood from Ruth's thesis that you did not clean all the lungworms from these sheep, that *Muellerius* and *Pneumostrongylus* were present.

REPLY BY MCGLINCHY: They were not found at necropsy.

REPLY BY HIBLER: Did you find first stage larvae?

REPLY BY RUTH MONSON: Prior to necropsy, there were some first stage larvae of *Pneumostrongylus*. The *Muellerius* were removed by the Tramisol.

REPLY BY HIBLER: Is it possible that the presence of *Pneumostrongylus*, which were not removed, and the presence of *Ostertagia* and *Trichuris* could have interfered with your physiological values?

REPLY BY MCGLINCHY: I think that in the case of eosinophilia, yes. I think in the case of bicarbonate, no. As I said, I consider these only trends and to be looked into more closely.

QUESTION BY GEORGE POST, CSU: The number of *Ostertagia* present, I think, was 4 *Ostertagia* on sheep #3; sheep No. 4 had 1. On number 3 we found no adult *Trichuris*, just occasional eggs on flotation. Somehow or another we couldn't find that worm. It wasn't a heavy parasitism at all here with *Ostertagia* and *Trichuris*. The *Pneumostrongylus* must have been in there in such low numbers we couldn't find them either. Concerning *Muellerius*, these sheep were put into isolation on April 10. They were kept in isolation until the 12th of March of the next year. Shortly after isolation they were given Tramisol. No *Muellerius* larvae were seen from mid-April until March of the next year and we couldn't find *Muellerius* on necropsy. So we're pretty sure Tramisol did an excellent job on *Muellerius capillaris*.

REPLY BY HIBLER: What species of *Pneumostrongylus* is common back there?

REPLY BY AL WOOLF, RACHELWOOD, PENNSYLVANIA: It's the one in white-tailed deer, *P. tenuis*.

REPLY BY HIBLER: I didn't know that *Pneumostrongylus tenuis* was normally found in domestic sheep. Therefore, I would wonder if the mouflon or the bighorn would be susceptible to it.

REPLY BY GEORGE POST: It's the one that's in white-tailed deer back there. Since they are raised in the same general vicinity with the white-tailed deer, they have this *Pneumostrongylus*. We never saw an adult, that's why I cannot say what species we are dealing with here. All we ever saw was a first stage larvae of *Pneumostrongylus* in the mouflon x bighorn cross. We assume that its source was the white-tailed deer.

REPLY BY WOOLF: We frequently find the adults in our deer, but we never have seen them in any of our sheep upon necropsy.

REPLY BY HIBLER: Where did you find the adults?

REPLY BY WOOLF: In the cranial wall.

REPLY BY POST: On our necropsies we have gone through the brain, the cranial case, as much of the spinal cord as we could get. We cut sections of spinal cord, but we saw no scarring on these animals, even though we knew they had *Pneumostrongylus*.

REPLY BY HIBLER: The point I am driving at here -- Sara's pretreatment evaluation found such and such a physiological value when these parasites were present and then upon treatment found a certain value. Then upon post-treatment with *Protostrongylus* found a value. Maybe I misunderstood, but a lungworm is a lungworm and I would expect in the case of *Pneumostrongylus* or *Protostrongylus*, since they are very closely related species, a very similar physiologic response on the part of the animal.

REPLY BY POST: This could be, except that you get very little lung damage from *Pneumostrongylus* and very low levels of first stage larvae.

REPLY BY HIBLER: Okay, then I might ask, "on an H and E section, how do you separate *Protostrongylus* and *Pneumostrongylus* larvae?" but I won't. What I'm driving at is, I would hate to try to evaluate a physiologic condition in an animal when lungworm is present and has been removed and then re-introduced. I'd hate to try to evaluate these levels.

REPLY BY POST: You mean you would hate to do this over a year's time?

REPLY BY HIBLER: Let's take this up after the meeting.