# Paramphistomosis in sheep; natural infection of lambs by *Calicophoron daubneyi*

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#### SUMMARY

In order to follow the course of natural infection by *Calicophoron daubneyi* in sheep, fourteen naïve lambs were allowed to graze a known infected pasture. Repeated blood and faeces samples were taken and every two weeks two lambs were necropsied. Tissue samples from the abomasum, duodenum and local lymph nodes were obtained for histological examination.

During the follow up, a softening of the faeces from D38 to D52 was the unique clinical sign observed. Significant hematological changes were noted in red blood cell, eosinophil, basophil and lymphocyte counts. Haemorrhagic lesions evident on D9 in the abomasum and duodenum were more marked on Days 38 and 52; varying numbers of immature paramphistomes were found during necropsies from D9 onwards. Histological examination revealed oedema and haemorrhages in the abomasum and duodenum, with infiltration by lymphocytes and eosinophils.

During the course of the natural infection by *C. daubneyi* in lambs, three phases were observed: firstly, an early eosinophilic non-specific reaction, secondly, an extension of parasite-induced lesions with a significant inflammatory reaction and thirdly a healing phase associated with parasite maturation. Unfortunately, the follow-up (80 days) was too short for determination of the prepatent period.

Keywords: *Calicophoron daubneyi*, sheep, natural infection

#### RÉSUMÉ

Paramphistomose chez le mouton: suivi d'une infestation naturelle d'agneaux par *Calicophoron daubneyi* 

Afin de suivre l'évolution d'une infestation naturelle par *Calicophoron daubneyi*, quatorze agneaux naïfs ont été introduits sur une pâture contaminée par ce parasite. Toutes les 2 semaines, des prélèvements sanguins et de matières fécales ont été réalisés et 2 agneaux ont été autopsies. Des examens histologiques ont été réalisés sur des échantillons de caillette, duodénum et nœuds lymphatiques.

Le seul signe clinique observé a été un ramollissement des matières fécales à J38 et J52. Les paramètres hématologiques suivants ont varié significativement : hématocrite, taux d'éosinophiles, de basophiles et de lymphocytes. Au niveau de la caillette et du duodénum, des lésions hémorragiques ont été relevées à J9 et étaient les plus marquées à J38 et J52. Un nombre variable de paramphistomes immatures a été observé dès J9. Les examens histologiques ont mis en évidence de l'œdème et des hémorragies dans la caillette et le duodénum, avec infiltration par des lymphocytes et des éosinophiles.

Ce suivi d'infestation naturelle a révélé trois phases: premièrement une réaction éosinophilique précoce non-spécifique, deuxièmement, une extension des lésions avec une réaction inflammatoire importante, troisièmement, une phase de cicatrisation avec maturation du parasite. Le suivi (80 jours) a été trop court pour déterminer une période prépatente.

## Mots-clé : *Calicophoron daubneyi*, agneaux, infestation naturelle

## Introduction

*Calicophoron daubneyi* is becoming more and more prevalent in French cattle [10; 18], sheep [1] and goats [17].

The incidence of this rumen fluke seems to be increasing in Europe and, recently, infections in cattle have been reported from Belgium [9], Italy [4], Spain [6], Ireland [16], Great Britain [8] and Portugal [2].

Although veterinary practitioners consider adult paramphistomes of little pathogenic significance, there are several reports of fatal damage related to immature worms in cattle and sheep in wet grazing areas [7, 8, 5, 11, 15].

At present, the normal course of natural infections is not well understood. It appears to differ from that of *Fasciola hepatica* even though both trematodes use the same principal intermediate host (*Galba truncatula*). This article reports a follow up study in a veterinary practice where natural infections occurred in lambs grazing on a pasture known to be heavily contaminated by C. *daubneyi* due to grazing in previous years by infected cattle.

## Materials and methods

#### STUDY AREA

This study was carried out in "Monts du Lyonnais" (East Central France, elevation: 510m) from 17 Aug to 23 Nov 2013. The mean annual rainfall is 774 mm; the monthly rainfall figures during the follow up from August to November were102, 54, 42 and 103 mm respectively and the mean temperatures varied from 20°C to 8°C, with a minimum of -4°C recorded once in November.

#### ANIMALS AND EXPERIMENTAL PROTOCOL

Fourteen 6-month old lambs were bought from two farms with no previous history of infection with *C. daubneyi*.

On Day -18 (17 Aug. 2012) faecal samples were collected from each lamb and, after weighing, they were injected intra-muscularly with doramectin (Zearl <sup>TM</sup>, Elanco; 200µg/ kg BW) and drenched with diclazuril (Vecoxan <sup>TM</sup>, Elanco; 1mg/kg BW). They were then housed and fed with grass silage until they went to pasture known to be infected by *C. daubneyi* and never previously grazed by sheep, on Day 0 (4 Sept). Infection of the pasture grazed by sheep during this experiment had been confirmed by the finding of many immature *C. daubneyi* at necropsy of a heifer which died after grazing in this pasture the previous year.

On D0, D9, D23, D38, D52, D66 and D80, lambs had blood and faeces samples taken. At each point, two lambs were removed and necropsied at the Departmental Veterinary Laboratory (F42600 Montbrison). These lambs had been ranked randomly for slaughter.

#### PARASITOLOGICAL AND BLOOD PARAMETERS

#### Fecal egg counts

Samples were collected directly from the rectum. Faecal egg counts were performed by a modified McMaster method using zinc sulphate (g: 1,3) which had a sensitivity of 20 eggs per gram (epg) of faeces.

#### **Blood parameters**

EDTA blood samples were collected for blood count using a Sysmex XN-2000 <sup>TM</sup> (Hematology System, Sysmex Europe GmbH, Nordestedt, Germany) and sera were stored at -20°C for total protein and albumin estimations with a Vet Test <sup>TM</sup> Chemistry Analyzer (IDEXX Laboratories).

These estimations were carried out on D0 (only from the 8 lambs which would still be still alive on D38), D38 and D66.

#### Necropsy and histological examination

Two lambs were necropsied on D0, D9, D23, D38, D52, D66 and D80. The abomasum and small intestine were carefully examined in order to detect immature worms and lesions. The abomasum, small intestine and mesenteric lymph nodes were weighed. Tissue samples were taken from the abomasum, duodenum (2 samples: at 25 and 50 cm from the pylorus), lymph nodes and any lesions observed in the liver, rumen, jejunum and ileum. Samples were formalin-fixed, paraffin-embedded and 4  $\mu$ m sections were stained with haematoxylin, eosin and saffron. The eosinophils in each sample were counted in 10 high power randomly chosen fields.

#### STATISTICAL ANALYSIS

Data were analyzed using mixed models to account for the repetition of measures on the same animals, except for the postmortem data. Sampling days were used as only categorical variables. Linear regressions were applied on all response variables, except the faeces consistency for which an ordered multinomial logistic regression was used. Cell counts and the mean hemoglobin concentration were logtransformed to assure a better fit of the model to the data.

#### Results

#### COPROSCOPY

No eggs of *C. daubneyi* were found at any time during the experiment

#### CLINICAL SIGNS AND DAILY WEIGHT GAIN

No clinical signs were observed during this study.

Because of early feeding changes experienced by the lambs, the first period considered for the daily weight gain (DWG) is D-13 to D9. From D-13 to D9, the DWG increased until D38 and then decreased and became negative after D52 (Figure 1).



FIGURE 1: Daily Weight Gain (grams per day).

Faeces appeared soft on D9 and from D38 to D52; normal faeces were observed from D66 onwards (Table 1).

#### HEMATOLOGY

Values of Mean Packed Cell Volume (PCV), Red Blood Cell Count (RBC) Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Volume (MCV) are given in Figure 2. These showed changes but remained within the normal reference ranges. PCV and RBC decreased on D9 and increased regularly thereafter. MCH and MCV stayed at low levels from D38.



FIGURE 2: Values of mean haematocrit (PCV), mean corpuscular volume (MCV), red blood cells (RBC), corpuscular haemoglobin (MCH) with 95% confidence interval.

Score:	1	2	3	4	5	Total samples
D0			2		11	13
D9		4	2	4	1	11
D23		1	1	5	3	10
D38		7	1			8
D52		4	1			5
D66					4	4
D80					2	2

TABLE I: Faeces score. 5: isolated droppings; 4: agglomerated droppings; 3: haped droppings; 2 soggy faeces; 1: liquid faeces

Day	Lymphocytes	Basophils	Eosinophils	Tissue eosinophils
D0	6,1 (5,3 , 7,0)	0,05 (0,03 , 0,08)	0,02 (0,01 , 0,04)	62 (30 , 129)
D9	4,2 (3,6 , 4,9)	0,03 (0,02 , 0,05)	0,05 (0,02 , 0,11)	34 (17 , 71)
D23	4,3 (3,7 , 5,0)	0,10 (0,06 , 0,18)	0,17 (0,07 , 0,41)	67 (33 , 138)
D38	5,3 (4,5 , 6,3)	0,06 (0,06 , 0,12)	0,06 (0,02 , 0,16)	144 (70 , 296)
D52	5,4 (4,5 , 6,4)	0,01 (0,01 , 0,03)	0,29 (0,10 , 0,85)	110 (53 , 225)
D66	3,6 (3,0 , 4,5)	0,12 (0,05 , 0,28)	0,62 (0,17 , 2,18)	100 (49 , 206)
D80	4,4 (3,4 , 5,8)	0,04 (0,01 , 0,13)	0,47 (0,09 , 2,61)	181 (88 , 373)

TABLE II: Lymphocytes, basophils and circulating eosinophils in % and tissue eosinophils with 95% confidence interval

Day	ТР	Albumin	Globulins	
D0	71,9 (66,8 , 77,0)	32,8 (30,0 , 35,5)	39,4 (35,1 , 43,7)	
D38	67,0 (61,9 , 72,1)	30,0 (27,3 , 32,7)	36,8 (32,5 , 41,0)	
D66	72,4 (65,6 , 79,1)	34,2 (30,5 , 38,0)	39,0 (34,1 , 43,8)	

TABLE III: Total protein, albumin and globulins (g/l) with 95% confidence interval

Lymphocyte, eosinophil and basophil numbers in circulating blood and tissue eosinophil counts are given in Table 2. They tended to evolve by waves.

For all these data, variations are shown according the time of infection.

#### PLASMA PROTEINS

The total plasma protein (TP), albumin (Alb) and globulin (Glob) levels decreased from D0 until D38 and then increased to reach levels similar to those recorded on D0, (Table 3).

#### NECROPSY FINDINGS

#### Abomasum

On D9 the mucosa was congested and slightly oedematous with visible white nodules of about 5 mm diameter; a small number of immature paramphistomes (less then  $500\mu$ m) were found on the mucosa.

On D23, hemorrhagic lesions and many 5 mm nodules were observed with moderate numbers of immature worms evident on the mucosa.

From D66 the lesions were less marked. The recorded relative abomasal weights were maximum from D38 to D66 (Figure 3).





FIGURE 3: Relative weights of abomasum, small intestine and mesenteric lymph nodes (in ‰ of body weight) with 95% confidence interval

#### Duodenum

Hemorrhagic lesions were evident as early as D9. Their intensity increased and became most marked on D38 and D52 with catarrhal enteritis and necrotic spots around immature worms visible through the gut wall (Figures 4 and 5). During the final month the lesions regressed and the duodenal wall became less thickened. Immature paramphistomes were present from D23 and several thousands were present on D38 and D52. These numbers had reduced dramatically by D66 and D80. Parasites and lesions were found along the first six meters of the small intestine but were more numerous in the first meter. The relative small intestine weights were maximal on D38 (Figure 3).



FIGURE 4: Small intestine of a lamb necropsied on D52. Haemorrhage and ulcers are visible through the peritoneal surface.



FIGURE 5: Duodenum of a lamb necropsied on D52. Immature paramphistomes are visible in the centre of necrotic dots.

#### Forestomachs

No visible lesions were found. Immature worms had arrived there by D38 and became progressively more

numerous from then on. From D52 the parasites measured 1,5-2mm length and were all immature.

#### Oesophagus

From D52 a few (2 to 10) immature paramphistomes were present on the oesophageal mucosa but there were no associated lesions.

#### Lymph nodes

From D9 to D52, the lymph nodes became more and more edematous, congested and haemorrhagic but during the last month, they appeared firmer. Their relative weights (Figure 3) had decreased by D23 then increased on D38 before showing a further decrease up to D80.

#### HISTOLOGY

#### Abomasum

On D9 and D23, the mucosa was oedematous and lightly infiltrated with lymphocytes and plasma cells forming small nodules in the mucosa. On D38 and D52 infiltration became moderate and multifocal with nodules containing lymphocytes, plasma cells and eosinophils. From D66 this infiltration decreased slightly and eosinophils became more numerous.

#### Duodenum

On D9 there were erosions of the epithelium and the lamina propria was moderately infiltrated with lymphocytes, plasma cells and eosinophils. A few multifocal haemorrhagic areas were evident on D23.

On D38 and D52, mucosal haemorrhages were marked with areas of necrosis. The mucosa was infiltrated with lymphocytes and plasma cells with local infiltrations of eosinophils. Immature worms were present in the mucosa (Figure 6).

From D66 the lesion intensity decreased and the inflammatory infiltration became more diffuse.

The tissue eosinophil counts decreased dramatically on D9 before showing a subsequent increase up to D38; they had then decreased by D66 before markedly increasing on D80 (Table 2).

#### Forestomachs

From D38, the mucosae were slightly oedematous and inflamed and on D80, the rumen papillae were irregular.



FIGURE 6: Immature paramphistome in the duodenum wall (D52).

## Discussion

Observations reported in this paper are similar to those described in naturally occurring fatal cases of paramphistomosis and in experimental infections. In ovine fatal cases, Mason et al [11] reported severe haemorrhagic enteritis of the duodenum with nodules visible on the mucosal surface, 3 weeks after being put out to graze a suspected contaminated pasture. At necropsy, they also observed adult worms in the rumen indicating that this was a reinfestation. In cattle congestive enteritis is described 5 weeks after first time grazing [5]. Lymphoplasmocytic infiltration with immature paramphistomes in the mucosa has been described at necropsies of grass-fed calves [7, 15] and lymphocytic and eosinophilic infiltration of the mucosa has also been observed in slaughtered sheep infested by various paramphistome species [20].

According to Bida & Schillhorn [3] sheep which died due to heavy *Calicophoron microbothrium* infestation also showed ulcers and oedema in the intestinal mucosa while cattle experimentally infected with C. *microbothrium* had catarrhal enteritis and mucosal corrugation [13].

This paper is the first report of a study of sheep naturally infected by immature *C. daubneyi*. The homogeneity of the macroscopic lesions observed in the two lambs necropsied at each time point was remarkable and our results allow a dynamic description of the course of a naturally acquired infection with *C. daubneyi* in lambs.

At the beginning of the follow up period, lambs were C. *daubneyi*-free but had previously experienced other parasitic infections, which probably explain the fact that the ingestion of metacercariae induced a rapid and marked nonspecific anamnestic-type eosinophilia. The only clinical sign during the first weeks was softening of the faeces on D9. In the case of reinfections, the clinical signs can be more serious leading

to severe weakness and occasionally death [11]. Nevertheless, in our trial, infection did not have any marked impact on the growth rates. The variations were statistically significant but can be explained by other factors such as feeding changes (in August and September) and climate (in November).

Our results show an early tissue eosinophil reaction followed by a subsequent important eosinophil recruitment and an increase in circulating eosinophils. It is well known that eosinophils can be involved in the destruction of helminth larvae [14] and tissue eosinophilic reactions have also been found in experimental nematode infections [21].

PCV and RBC counts decreased during the first 10 days and then increased to a higher level. After a single experimental infection of cattle with C. *microbothrium*, Mavenyengwa et al. [12] found changes in these parameters which were related to the numbers of larvae administered. In our study the numbers of ingested metacercariae were not known and the infection was continuous during the course of the experiment.

In this study, D38 seemed to be a key point in the evolution of the lamb-parasite interaction with regard to clinical impact with softening of the faeces in all eight surviving animals; at the same time the relative weights of the abomasa, small intestines and lymph nodes were maximal and decreased TP and Albumin levels were recorded.

From D38 to D52 the tissue lesions and the inflammatory response became more marked. During this time the worms progressively migrated to the forestomachs with a corresponding decrease in their numbers in the small intestine.

From D66 the duodenum mucosal lesions were less marked and there were fewer immature parasites. The size of the worms present in the different compartments was similar and was higher than those found on D9 suggesting that there was little new establishment of paramphistomes after the first weeks.

Two hypotheses can explain this: (1) a decrease in numbers of metacercariae ingested for whatever reason; (2) the host reaction, in particular the high level of tissue and circulating eosinophils which might have affected parasite establishment. As the experiment was conducted under field conditions it is impossible to confirm either of these two hypotheses although challenge infections with *C. microbothrium* have been shown to produce lower establishment rates than a primary infection of susceptible animals (13). However it is likely that previous exposure and level of challenge will both have an influence on the response to infection with paramphistomes.

The results of this study did show that the first two months following exposure are the most important in terms of the development of this parasitic infection although clinical signs in the lambs which were well-fed over the study period, were minimal.

In the rumen the only macroscopic lesions were irregular papillae. Histologically the reticulum and rumen were slightly oedematous and inflamed once immature worms were present. These lesions were also described in fatal clinical cases in cattle [7] and sheep [11]. C. *microbothrium* and *Carmyerius marchandi* also induced similar rumen lesion in healthy cattle [19]. There was no evidence that these lesions, with C. *daubneyi*, had any clinical or metabolic impact.

## Conclusion

The monitoring of naïve lambs exposed to natural infection by grazing a *C. daubneyi* infected area, showed three phases in the establishment of *C. daubneyi*: firstly, an early nonspecific eosinophilic reaction during the period of infection with juvenile worms in the abomasum and duodenum where lesions were observed as early as 9 days after exposure; secondly, an extension of parasite-induced lesions with an associated, significant inflammatory reaction and thirdly a healing phase associated with parasite maturation. These findings might help explain lesions which can be found after first exposure of ruminants to paramphistomes and further experiments are planned to study the response to reinfection and determine the duration of the prepatent period.

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