Onderstepoort J. vet. Res. 38 (3), 191–198 (1971)

STUDIES ON *DICTYOCAULUS FILARIA*

III. THE MIGRATION OF THE IMMATURE STAGES APPLIED TO AN ANTHELMINTIC TEST

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ABSTRACT

REINECKE, R. K., COLLINS, H. MARIA & ANDERSON, P. J. S. Studies on *Dictyocaulus filaria*. III. The migration of the immature stages applied to an anthelmintic test. *Onderstepoort J. vet. Res.*, 38 (3), 191–198 (1971).

The larvae of *Dictyocaulus filaria* (Rudolphi, 1809) develop to the 5th stage in the mesenteric lymph nodes within 6 to 8 days of infestation. The migration of the 5th stage to the lungs commences on the 7th or 8th day and is almost complete by the 13th day. The adult females start laying eggs in the bronchi from the 28th day.

Controlled anthelmintic tests on two groups of sheep are described. Levamisole was dosed intraruminally at 7,5 mg/kg. It was tested against the 3rd and 4th stage larvae in the lymph nodes in the 1st group and against the 5th stages in the lungs in the 2nd group of animals. In both cases data assessed by the non-parametric statistical method showed that levamisole could be classified in Class B, i.e. it was more than 60 per cent effective in more than 60 per cent of the treated flock.

INTRODUCTION

The migratory pattern of the parasitic life-cycle of *Dictyocaulus filaria* (Rudolphi, 1809) was studied in detail by Anderson & Verster (1971b). They showed that the larvae reached the mesenteric lymph nodes within 18 hours of infestation, and that the worms started migrating to the lungs from the 7th or 8th day onwards. Thereafter migration proceeded rapidly, most of the worms being recovered from the lungs 14 days after infestation (Fig. 1). Adults were present after the 28th day.

Anthelmintic trials described in this paper were based on these observations and two groups of sheep were used. In one group each animal was infested daily for 8 days to test the efficacy against the 3rd and 4th stage larvae in the lymph nodes. The other group was infested every 3rd day from the 9th to the 30th day to assess efficacy against the immature 5th stages in the lungs.

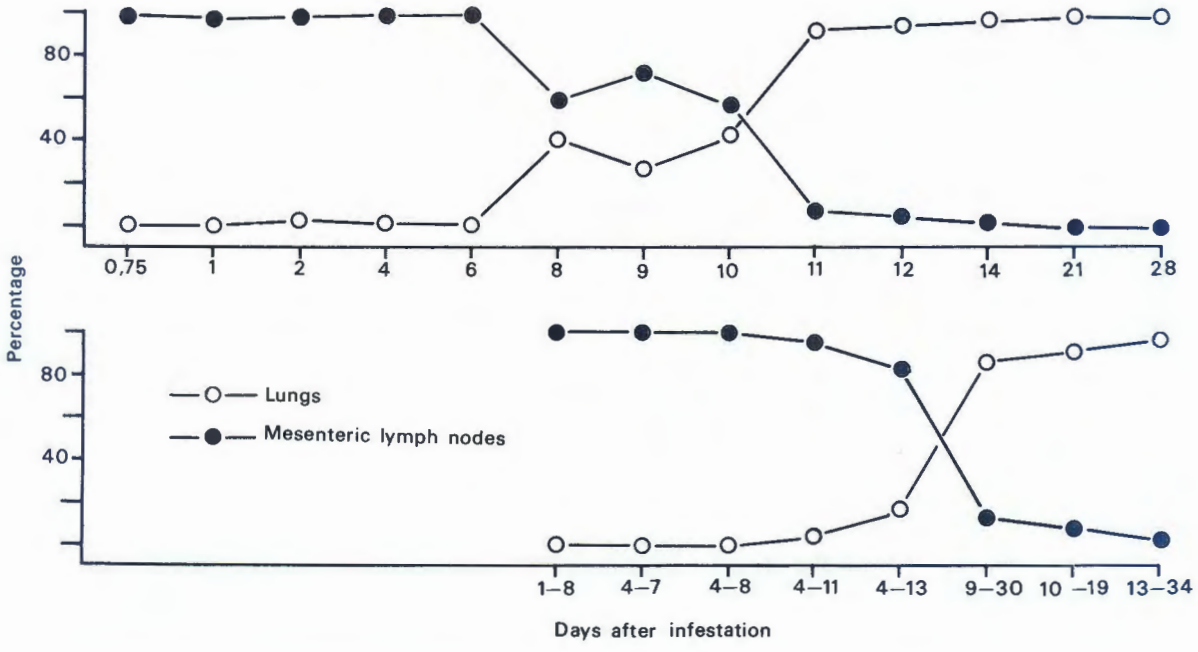

FIG. 1 The migration of *D. filaria* expressed as a percentage. Upper graph Anderson & Verster (1971b). Lower graph these data.

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Received 26 March 1971 - Editor

TABLE 1 *Experimental design*

Day	No. of infective larvae of *D. filaria* dosed to each sheep	
	Group 1	Group 2
—30	—	155
—27	—	156
—24	—	208
—21	—	159
—21	—	Sheep 41: Larval viability control Day —30 to Day —21 withdrawn after dosing
—18	—	Sheep 42: Larval viability control Day —18 to Day —9 dosed with other sheep
—18	—	175
—15	—	177
—12	—	178
—11	—	Killed Sheep 41: Larval viability control Day —30 to Day —21
— 9	—	143
— 8	117	—
— 7	162	—
— 6	184	—
— 5	230	Killed Sheep 42: Larval viability control Day —18 to Day —9
— 5	—	
— 4	208	
— 4	Sheep 44: Larval viability control Day —8 to Day —4 withdrawn after dosing	—
— 3	168	—
— 3	Sheep 43: Larval viability control Day —3 to Day —1 dosed with other sheep	—
— 2	176	—
— 1	159	—
Total	1 404	1 351
0	Killed Sheep 1-9 inclusive Controls Treated Sheep 10-20 inclusive with levamisole at 7,5 mg/kg intraruminally. Killed Sheep 44: Larval viability control Day —8 to Day —4	Killed Sheep 21–29 inclusive Controls Treated Sheep 30-40 inclusive with levamisole at 7,5 mg/kg intraruminally
+ 3	Sheep 16 treated on Day 0 died overnight. Killed Sheep 10-15 and 17-20 inclusive treated on Day 0. Killed Sheep 43: Larval viability control. Day —3 to Day —1	—
+ 4	—	Killed Sheep 30-40 inclusive: treated on Day 0

MATERIALS AND METHODS

Forty-four Dorper yearling sheep were dosed with 26 ml of thiabendazole 13,5% m/v, i.e. a dosage rate varying from 76 to 139 mg/kg. They were fed and housed under worm-free conditions. Two days later they were divided into two groups of 22 sheep.

The animals were dosed with infective larvae of *D. filaria* as indicated in the experimental design (Table 1). On Day 0, 11 sheep in each group were treated with levamisole at 7,5 mg/kg intraruminally. In each group nine controls were killed on the day of treatment. In addition two sheep were dosed with larvae for a limited period to determine the infectivity of these larvae. These animals (Sheep 41, 42, 43 and 44) were designated as larval viability controls. The treated sheep in Group 1 were killed 3 days and those in Group 2 4 days after treatment.

The methods for the recovery of the worms were similar to those described by Anderson & Verster (1971a). The following lymph nodes from each sheep were manually removed and placed in a glass jar: hepatic, cranial mesenteric and right colic. Similarly the caudal mediastinal and bronchial lymph nodes and the lungs were placed in separate labelled containers.

The lymph nodes were cut with scissors into pieces 2 to 3 mm in width and placed on a flat gauze surface in a modified Baermann apparatus which was constructed as follows: Nylon gauze with apertures 220 micron was placed over the top of a 2 l beaker. A steel wire ring which acted as a spring was placed on top of the gauze and pushed down into the beaker for a distance of 5 cm. The beaker was filled with saline to 2 cm above the flat gauze surface and then placed in a waterbath at 40°C for 3 hours.

The trachea and bronchi were cut open with bowel scissors and washed into a bucket marked "lung washings". The lungs were then cut into pieces 1 cm thick, which were placed in a galvanized steel trap containing saline and submerged under a piece of nylon gauze weighed down with a wire basket. This specimen was marked "lung snips" and was placed in a waterbath at 40°C for 3 hours (Reinecke, 1967).

The lung washings were placed in a waterbath and the worms killed by heating to 60°C. Thereafter they were fixed with formalin and sieved through a 100 mesh to the linear inch (25,4 mm) sieve (Endecott). The residue on the sieves was washed into a labelled glass jar and formalin added to 1/10 of the final volume.

The filtrate of the lymph nodes was heated to 60°C, fixed with formalin and then filtered through a 400 mesh to the linear inch (25,4 mm) sieve (Endecott). The residue on the sieve was placed in a labelled glass jar and formalinized.

The wire basket and nylon gauze were removed from the trap containing the lung snips and the filtrate poured into a bucket. The lung snips were placed on a

coarse sieve with apertures of 2 mm and adherent worms washed into the bucket containing the filtrate with a strong stream of water. Thereafter the filtrate in the bucket was treated as described for the lymph nodes above.

Microscopic examination for the recovery of the worms was similar to that previously described by Reinecke (1967). The larval stages were identified using the description of Douvres & Lucker (1958) for *Dictyocaulus viviparus* (Bloch, 1782).

Results

Group 1: One- to eight-day-old-worms:

The results are summarized in Table 2.

Two-hundred and forty-seven worms were recovered *post-mortem* from Sheep 44 and 26 worms from Sheep 43, which represent 25,7 per cent and 5,2 per cent respectively of the infective larvae dosed. This result indicates that the first five larval doses, i.e. from Day 8 to Day 4, contained more viable infective larvae than those dosed from Day 3 to Day 1.

No worms were recovered from Sheep 8. The sample was not formalinized at the time of bottling, and the worms either underwent autolysis or were destroyed by fungi. Anderson & Verster (1971 a) noted that predacious fungi destroyed the pre-parasitic stages of *D. filaria;* it is possible that the early parasitic stages are similarly affected.

Table 2 *Group 1*: D. filaria *recovered at autopsy*

Group	Sheep No.	Organ	Stage of development			Total
			**L_3	**L_4	**5	
Larval viability control Day —8 to Day —4						
Killed on Day 0	44	Mesenteric lympth nodes	95	121	31	247
Larval viability control Day —3 to Day —1						
Killed on Day + 3	43	Mesenteric lymph nodes	8	18	0	26
Controls:						
Killed on Day 0	1	Mesenteric lymph nodes	25	156	42	223
„ „ „ „	2	„ „ „	43	378	9	430
„ „ „ „	3	„ „ „	34	219	9	262
„ „ „ „	4	„ „ „	32	228	38	298
„ „ „ „	5	„ „ „	21	172	58	251
„ „ „ „	6	„ „ „	6	145	13	164
„ „ „ „	7	„ „ „	35	232	58	325
„ „ „ „	*8	All organs	0	0	0	0
„ „ „ „	9	Mesenteric lymph nodes	19	89	7	115
		mean	23,8	179,8	26,0	229,9
		s.d.	±14,1	±105,0	±23,0	±125,3
Treated on Day 0 with levamisole at 7,5 mg/kg intraruminally						
Killed on Day + 3	10	Mesenteric lymph nodes	0	80	4	84
„ „ „ „	11	„ „ „	0	16	0	16
„ „ „ „	12	„ „ „	0	16	0	16
„ „ „ „	13	„ „ „	0	8	1	9
„ „ „ „	14	„ „ „	0	11	2	13
„ „ „ „	15	„ „ „	0	102	18	120
	(a)	„ „ „	0	19	3	22
Died	16	Lung washings	1	0	0	1
		Total	1	19	3	23
		Mesenteric lymph nodes	0	137	28	165
Killed on Day + 3	17	Lung snips	0	17	5	22
		Total	0	154	33	187
„ „ „ „	18	All organs	0	0	0	0
„ „ „ „	19	Mesenteric lymph nodes	0	84	13	97
		Mesenteric lymph nodes	0	60	5	65
„ „ „ „	20	Lung washings	8	0	0	8
		Total	8	60	5	73
		mean	0,8	50	7,0	58
		s.d.	—	±49,7	±10,2	±59,5

*No formalin was added to this specimen and fungi were present in the bottle. These were probably predacious fungi which destroyed the worms.

**L_3 = third stage larvae; L_4 = fourth stage larvae; 5 = fifth stage.

(a) Sheep 16 died overnight, i.e. between Day + 2 and Day + 3.

TABLE 3 *Group 2:* D. filaria *recovered at autopsy: Controls*

Group	Sheep No.	Organ	Stage of development		Total
			L_4	5	
Larval viability control Day —32 to Day —21					
Killed on Day —11	41	Mesenteric lymph nodes	7	6	13
		Lung washings	0	63	63
		Lung snips	0	82	82
		Total	7	151	158
Larval viability control Day —18 to Day —9					
Killed on Day —5	42	Mesenteric lymph nodes	(8)28	2	30
		Lung washings	0	1	1
		Lung snips	3	2	5
		Total	(8)31	5	36
Controls					
Killed on Day 0	21	Mesenteric lymph nodes	21	27	48
		Lung washings	0	190	190
		Lung snips	5	175	180
		Total	26	392	418
„ „ „	22	Mesenteric lymph nodes	1	19	20
		Lung washings	0	124	124
		Lung snips	0	99	99
		Total	1	242	243
„ „ „	23	Mesenteric lymph nodes	4	27	31
		Lung washings	0	44	44
		Lung snips	0	131	131
		Total	4	202	206
„ „ „	24	Mesenteric lymph nodes	12	41	53
		Lung washings	0	258	258
		Lung snips	0	154	154
		Total	12	453	465
„ „ „	25	Mesenteric lymph nodes	3	26	29
		Lung washings	0	2	2
		Lung snips	0	14	14
		Total	3	42	45
„ „ „	26	Mesenteric lymph nodes	2	11	13
		Lung washings	0	39	39
		Lung snips	0	26	26
		Total	2	76	78
„ „ „	27	Mesenteric lymph nodes	1	20	21
		Lung washings	0	58	58
		Lung snips	0	78	78
		Total	1	156	157
„ „ „	28	Mesenteric lymph nodes	5	39	44
		Lung washings	0	107	107
		Lung snips	0	112	112
		Total	5	258	263
„ „ „	29	Mesenteric lymph nodes	0	19	19
		Lung washings	0	76	76
		Lung snips	0	55	55
		Total	0	150	150
		mean	6,0	219,0	225
		s.d.	±8,3	±135,7	±142,0

TABLE 3 (Continued) *D. filaria* recovered at autopsy: Treated

Group	Sheep No.	Organ	Stage of development		Total
			L_4	5	
Treated on Day 0 with levamisole at 7,5 mg/kg intraruminally					
Killed on Day +4	30	Lung washings	0	10	10
		Lung snips	0	22	22
		Total	0	32	32
,, ,, ,,	31	Lung washings	0	*110	110
		Lung snips	0	**93	93
		Total	0	203	203
,, ,, ,,	32	Lung washings	0	***1	1
		Lung snips	0	4	4
		Total	0	5	5
,, ,, ,,	33	Lung snips	0	5	5
,, ,, ,,	34	Mesenteric lymph nodes	0	6	6
		Lung washings	0	7	7
		Lung snips	0	1	1
		Total	0	14	14
,, ,, ,,	35	Lung washings	0	38	38
		Lung snips	0	24	24
		Total	0	62	62
,, ,, ,,	36	Lung washings	0	12	12
		Lung snips	0	16	16
		Total	0	28	28
,, ,, ,,	37	Mesenteric lymph nodes	1	0	1
		Lung washings	0	3	3
		Lung snips	0	2	2
		Total	1	5	6
,, ,, ,,	38	Lung washings	0	27	27
		Lung snips	0	7	7
		Total	0	34	34
,, ,, ,,	39	Lung washings	0	23	23
		Lung snips	0	32	32
		Total	0	55	55
,, ,, ,,	40	Mesenteric lymph nodes	4	0	4
		Lung washings	0	6	6
		Total	4	6	10
		mean	0,4	44,4	41,3
		s.d.	—	±57,5	±57,1

*Including 4 adult females
**Including 2 adult females
***1 Adult female

In the other control animals all the worms were confined to the mesenteric lymph nodes and the numbers recovered ranged from 115 to 430. Most of them were 4th stage larvae, but 3rd stage larvae and 5th stage worms were present in moderate numbers.

When the treated sheep were killed the surviving worms varied from 4 to 11 days in age. The majority of the remaining worms were still in the mesenteric lymph nodes. Migration to the lungs, however, had started, as a few worms were recovered from this site in three sheep (Table 2).

Most of the worms were 4th stage larvae but some 5th stage worms were present. Third stage larvae were recovered from the lungs of Sheep 16 and 20, which

contained 1 and 8 respectively, but the other 9 sheep had no worms in this stage. Sheep 18 was negative.

Group 2: Nine- to thirty-day-old-worms:

The results are summarized in Table 3.

Considerably more worms were recovered from Sheep 41, the Day 30 to Day —21 viability control, than from Sheep 42, the viability control for Day 18 to Day 9. This represents the development of 23,3 per cent and 5,3 per cent respectively of the infective larvae dosed. Either the initial larval doses contained more viable infective larvae than those dosed during the latter period, or the worms which were 7, 10 and 13 days old at slaughter were actively migrating from the lymph nodes to the lungs and were therefore not recovered.

With one exception (Sheep 25), the worm burdens in the controls followed a similar pattern: in 8 of the 9 controls, counts ranged from 78 to 465; 5th stage worms constituted 83,2 to 91,7 per cent of the total worm burdens in the lungs and 7,7 to 14,2 per cent of those of the mesenteric lymph nodes. In Sheep 21 1,2 per cent of the worms recovered were 4th stage larvae in the lungs. Elsewhere this stage was either absent or confined to the mesenteric lymph nodes and did not exceed 5 per cent of the worms present.

Sheep 25 differed from the other controls. Only 45 worms were recovered and they were distributed as follows: 4th stage larvae in the lymph nodes constituted 6,6 per cent; 5th stage worms 57,7 per cent and 35,5 per cent in the mesenteric lymph nodes and lungs respectively.

The treated sheep were killed 4 days after the controls, when the age of the surviving worms varied from 13 to 34 days. In 7 of the 11 sheep all the worms, and in the other 4 sheep 57,1 to 83,3 per cent of them, were in the 5th stage in the lungs. Included with these were 1 and 6 adult females with embryonated eggs in their uteri which were recovered from Sheep 32 and 31 respectively. In Sheep 34 the mesenteric lymph nodes contained 42,8 per cent in the 5th stage; in Sheep 37 and 40 16,6 per cent and 40 per cent of the remaining total worm burden were 4th stage larvae.

Discussion

The rate of migration of *D. filaria* in these trials confirms the observations of Anderson & Verster (1971b). Either with single infestations of 10 000 to 15 000 or with multiple regular doses of infective larvae as in this case, the parasites migrate to the lymph nodes and remain there for 8 days. Thereafter mass migration to the lungs occurs and by the 13th day only a minor fraction remains in the lymph nodes.

The various larval stages were identified from the descriptions of those of *D. viviparus* by Douvres & Lucker (1958). Although this reference is brief, the essential morphological features of each developmental stage are enumerated and it was possible to recognize similar characteristics in *D. filaria*. Some differences for *D. filaria* should be emphasized.

Very few 4th stage larvae are present in the lungs. The 4th moult takes place in the mesenteric lymph nodes and the 5th stage migrates to the lungs. The males, although minute, have a fully formed bursa with bursal rays and weakly sclerotized spicules. Kauzal (1933) illustrates bursal rays in *D. filaria* recovered from the bronchus of a guinea pig 8 days after infestation, but labels it a 4th stage larva, because the ventral rays and spicules are absent. In Fig. 2 he illustrates a 5th stage worm, not a 4th stage larva, because the bursal rays are already present, and they are not surrounded by the cuticle of the 4th stage.

It is difficult to distinguish between the 4th moult and early 5th stage female. In *D. viviparus,* Douvres & Lucker (1958) describe the primordial vulvar lips present in the 4th moult; although the genital primordium is elongated it is not differentiated; in the 5th stage there is a vulvar opening but the organs of reproduction are still undifferentiated. In *D. filaria* worms with vulvar lips indicating the 4th moult, are classified as 4th stage larvae. The presence of a lumen in these organs is indicated by a distinct line in the ovijector which may extend to the adjacent uterine horns; this is the 5th stage female. Although the 5th stage female is recognizable by the 8th day, i.e. immediately prior to migration from the lymph nodes, many males have already reached the 5th stage by the 6th day. It is clear that the majority develop to the 5th stage in the lymph nodes.

It is convenient to define the 5th stage as a worm that has completed the 4th moult but has not yet reached sexual maturity. After fecundation the adult female is readily recognised by the presence of embryonated eggs in the uterus. Adult males are recognized by the degree of pigmentation of the spicules. The presence of spermatozoa would be a better criterion but they are more difficult to identify.

If it is accepted that the 4th moult of *D. filaria* takes place from the 6th to the 8th day and that the early 5th stage worms migrate to the lungs, it follows that the development to the 4th moult is very rapid; the larval stages are, therefore, confined to the lymph nodes. The 5th stage is present for a longer period than the larvae and the greater part of its development occurs in the lungs. The first embryonated eggs are noted after a further 3 weeks and the prepatent period is thus 28 to 30 days.

In this anthelmintic trial a distinction has been made between the larval stages up to and including the 4th moult in the lymph nodes and the immature adult or 5th stage in the lungs. In Group 1 no attempt was made to differentiate between 3rd and 4th stage larvae; this may or may not be of practical significance.

The anthelmintic test against 3rd and 4th stage larvae could be improved. One Day 0 control must be slaughtered to confirm the presence of 3rd and 4th stage larvae in the lymph nodes on the day of treatment. The remaining controls as well as the treated sheep should be slaughtered 7 or 8 days later. This would facilitate the identification of the worms which will be considerably larger 5th stages in the lungs. With this modification it would be unnecessary to recover and recognize the minute larval stages in the mesenteric lymph nodes.

Earlier studies on the life-cycle by Anderson & Verster (1971b) were confirmed by the results obtained in Group 2 (Fig. 1). Between the 9th and 13th day the worms are either in the mesenteric lymph nodes, or actively migrating, or have arrived in the lungs (see Sheep 21 to 29, Table 3). From 13 days onwards they are definitely in the 5th stage and, with rare exceptions in the lungs (see Sheep 30 to 40, Table 3). The design of a trial against 5th stages and adults could be modified so that sheep are infested every 2nd or 3rd day from Day 42 to Day 14. This would limit the test to the 5th and adult stages in the lungs and would facilitate assessment of the data by the non-parametric method described below.

Wilson (1970) challenged vaccinated goats and non-infested controls with massive doses of infective larvae of *D. filaria*. The goats were killed at various intervals

after challenge and both the mesenteric lymph nodes and lungs examined for worms *post mortem*. Five days after challenge all the larvae were in the lymph nodes; by the 10th day more than 91 per cent and after 15 days 97,8 to 100 per cent of the worms had migrated to the lungs. His trials showed a similar worm migration to that in sheep observed by Anderson & Verster (1971b) and confirmed in these trials.

Another method of testing anthelmintic efficacy is the critical test described by Vodrazka (1960). A complete tracheotomy is carried out and the number of worms expelled compared with those found at autopsy. Düwel (1963) is in favour of this critical test in assessing efficacy against adult *D. viviparus,* because there is a great variation in the numbers of worms recovered from experimentally infested calves. *D. filaria* shows a similar variation in the number of worms recovered. A controlled test is clearly essential for the assessment of efficacy against the histotrophic stages in the lymph nodes. A controlled test can also be used for the 5th and adult stages of *D. filaria,* providing the results are assessed by the non-parametric method.

Results are assessed by using the median rather than the mean to indicate the worm burdens of the controls. Initially, Groeneveld & Reinecke (1969), proposed a reduction in worm burdens on the lower limit of the median of at least 80 per cent, in at least 80 per cent of the treated flock. Simulation studies carried out by C. J. Clark, Imperial Chemical Industries, Macclesfield, England (1969 in a personal communication), however, showed that more than 80 per cent of the treated flock could be estimated by multiplying the control median by 0,25 rather than by 0,2 and by allowing one of the 11 results of the treated sheep to exceed the reduced median. This amounts to carrying out the estimations at the 75 per cent: 75 per cent level; but in spite of this a compound has little chance of causing 80 per cent or less reduction in 80 per cent or less of the treated flock. This is defined as Class A.

In Class B there is more than 60 per cent reduction in more than 60 per cent of the treated flock. The control median is multiplied by 0,4 and 3 of the 11 results on the reduced control median in the treated sheep can exceed this figure.

In Class C there is more than 50 per cent reduction in more than 50 per cent of the treated flock. The control median is multiplied by 0,5 and 4 of 11 results in the treated group can exceed the reduced control median.

Compounds are classified as X or ineffective if they do not comply with the requirements of Class C.

Thus in Group 1 the control median

251 (0,25) = 62,75
251 (0,4) = 100,4

and in Group 2 the control median

206 (0,25) = 51,5
206 (0,4) = 82,4

In Group 1 the compound cannot be classified as Class A because only 6 of 11 results comply; similarly in Group 2 only 8 of 11 results comply.

In both groups, however, 9 and 10 results respectively out of the 11 treated sheep are less than the control median (0,4) for the 60 per cent: 60 per cent level; the compound therefore fulfils the requirements for Class B, as it is more than 60 per cent effective against 3rd and 4th stage larvae and 5th stage worms in more than 60 per cent of the treated flock.

Summary

A controlled anthelmintic test with levamisole at 7,5 mg/kg was carried out on two groups of sheep infested experimentally with infective larvae of *D. filaria*. In one group the worms were 1 to 8 days old and in 3rd, 4th, and early 5th stage in the mesenteric lymph nodes. In the other there were 5th stage worms in the lungs 9 to 30 days old at treatment.

Acknowledgements

We wish to thank Dr. J. Phillip of A. S. Ruffel (Pty) Ltd. sincerely for the assistance of his technical staff, Messrs. F. Erasmus and S. Broderyk, as well as the supplies of infective larvae and levamisole. Dr. Anna Verster and Dr. J. A. van Wyk helped us considerably with the manuscript. The assistance of Dr. W. P. van Aardt, Mrs. Jeanne Anderson and Messrs. J. Pretorius and J. Theron is gratefully acknowledged.

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Printed in the Republic of South Africa for the Government Printer, Pretoria, by Heer Printing Co. (Pty) Ltd., Pretoria