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IMPROVED METHODS FOR THE RECOVERY OF PARASITIC NEMATODES AT AUTOPSY

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INTRODUCTION

The minute larval stages of parasitic nematodes are well concealed in large masses of ingesta and gut wall. This paper describes improved methods for their recovery and concentration at autopsy.

TABLE 1.—*Experimental design*

Day	No. of infective larvae dosed to each sheep		
	O. columbianum	*O. circumcincta*	*T. colubriformis*
−58	30	—	—
−55	30	—	—
−51	30	—	—
−48	30	—	—
−44	30	—	—
−41	30	—	—
−36	30	150	150
−34	30	150	150
−30	30	150	150
−28	30	150	150
−26	30	150	150
−21	(*a*) 0	50	50
−19	0	50	50
−16	0	50	50
−13	0	50	50
−9	50	50	50
−8	30	100	100
−7	30	100	100
−6	30	100	100
−5	30	100	100
−4	30	100	100
−3	30	100	100
−2	30	100	100
−1	30	100	100
TOTAL	620	1,800	1,800

0 { Slaughtered Day 0 Control Sheep 1
Dosed Sheep 5, 6, 7 and 8 with Casella Anthelmintikum at 300 mg/Kg intra-ruminally
Dosed Sheep 9, 10, 11 and 12 with Casella Anthelmintikum at 450 mg/Kg intra-ruminally

+6 { Slaughtered Day+6 Controls: Sheep 2, 3 and 4
Slaughtered Treated Groups: Sheep 5 to 12 inclusive

(*a*) Pure cultures of *O. columbianum* not available from Day −21 to Day −13

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METHODS FOR THE RECOVERY OF PARASITIC NEMATODES AT AUTOPSY

EXPERIMENT 1: A MODIFIED BAERMANN APPARATUS

Materials and Methods

1. Twelve weaned Dorper (Dorset Horn × Black Head Persian) sheep, born, reared and maintained worm-free were used. They were dosed *per os* with infective larvae of *Oesophagostomum columbianum* (Curtice, 1890), *Ostertagia circumcincta* (Stadelman, 1894) and *Trichostrongylus columbriformis* (Giles, 1892) using the methods described by Reinecke, Horak & Snijders (1963).

2. The experimental design is summarized in Table 1. From Day−21 to Day−13 infective larvae of *O. columbianum* were not available for infestation.

3. On Day−1 nine 12-inch diameter brass sieves were thoroughly washed and placed in shallow plastic photographic trays (16 × 12·5 × 2 in.) to which 0·85 per cent NaCl solution was added until the mesh was covered with 1 to 2 mm fluid. Air bubbles trapped under the mesh were sucked off with a rubber tube connected via a vacuum flask to a negative pressure water pump. They were stacked in two incubators at a temperature of 37·5°C and left overnight.

4. Sheep 1 was slaughtered on Day 0, the abdominal cavity was opened and tight double ligatures were tied at the following points:—

(*a*) Between the omasum and reticulum and at the pylorus.

(*b*) The ileo-caecal valve.

(*c*) The colon ventral to the tip of the caecum, where an imaginary line from the tip of the caecum to the outer coil of the *ansa spiralis* bisects the colon.

(*d*) The rectum just anterior to the anus.

After stripping the mesentery the gut was cut between the double ligatures into four portions, which were handled separately.

5. Each portion was opened into a separate bucket and the wall washed with saline at 37·5°C. The previously prepared sieves were removed from the incubator and specimens placed on them as follows:—

(*a*) The ingesta from the abomasum and small intestine were each placed on 100 mesh sieves while those from the caecum plus the *ansa spiralis*, and from the descending colon plus the rectum were placed on two separate 44 mesh to the linear inch sieves. The four specimens were then incubated for three hours at 37·5°C.

(*b*) The abomasal and gut wall were cut into 1 to 2 cm pieces, placed on separate sieves with a gauge varying from 6 to 44 mesh to the linear inch and incubated at 37·5°C for six hours.

6. *Ingesta:* After incubation the sieves were lifted out of the plastic trays, the filtrate (in the trays) and residue (on the sieves) being kept separate.

(*a*) *Filtrate:* This was poured into labelled glass jars and stored at 4°C overnight. The supernatant was then discarded and the worms in the sediment killed by heating to 60°C. The suspension was washed through 400 mesh to the linear inch sieves, the material on the sieves collected and an equal volume of 10 per cent formalin added to it.

(*b*) *Residue:* The ingesta were washed off the sieves into buckets and the worms were killed by the addition of 3 ml of concentrated* iodine solution per litre, mixing it vigorously with the suspension as it was added. Subsequent sieving, collection and fixing of the worms were carried out by the method described under (*a*) Filtrate.

7. The filtrate from the gut wall was treated in the same manner as the filtrate from the ingesta.

The residue from the gut wall was placed in glass vessels and kept at 4°C overnight. The following day these residues were placed in a solution containing 10 gm pepsin and 30 ml HCl per litre of water. A quantity of 3 to 5 ml pepsin solution per gram of gut was added and the resultant suspension incubated at 45°C for six hours until the gut was digested. The worms were killed with concentrated iodine, sieved, collected and formolized as described in (6) above, and the results have been tabulated with those of the residue of the ingesta.

8. To facilitate their detection under the stereomicroscope, worms were stained strongly with concentrated iodine solution. The entire specimen was examined and the worms recovered from it were transferred to labelled specimen bottles containing a solution made up as follows:—

Formalin	350 ml
Triethanolamine	100 ml
50 per cent Picric acid powder	10 gm

Water added to produce a final volume of 5 litres.

This solution decolorized and preserved the worms, which were subsequently transferred to slides for examination with a standard microscope.

9. On Day 0 eight sheep were dosed intra-ruminally with Casella Anthelmintikum 10,001, four at 300 mg/Kg and four at 450 mg/Kg (Table 1).

*The concentrated iodine solution was made by dissolving 16 to 20 lb KI in as little distilled water as possible, adding 10 lb I and then water to produce a final volume of 10 litres.

TABLE 2.—*Worms recovered from Sheep* 1 *post mortem*

Species	Stage	Site	Number
O. circumcincta	Third	Filtrate	11
		Residue	0
	Fourth	Filtrate	21
		Residue	3
	Fifth	Filtrate	2
		Residue	3
	Adult	Filtrate	115
		Residue	147
T. colubriformis	Third	Filtrate	26
		Residue	0
	Fourth	Filtrate	39
		Residue	0
	Fifth	Filtrate	90
		Residue	0
	Adult	Filtrate	772
		Residue	113
O. columbianum	Fourth	Filtrate	43
		Residue	7
	Fifth	Filtrate	2
		Residue	84
	Adult	Filtrate	3
		Residue	88

Results (*see Table* 2)

With the exception of 113 adults (10·9 per cent) out of a total burden of 1,040 worms all stages of *T. colubriformis* were confined to the filtrate. Although the filtrates contained most of the third and fourth stage larvae of *O. circumcincta* and the fourth stage larvae of *O. columbianum*, the fifth stage and adult worms of these two species were more common in the residue.

TABLE 3.—*Worms recovered post mortem*

Group	Sheep No.	*O. circumcincta*					*T. colubriformis*					*O. columbianum*			
		Stage of development				Total	Stage of development				Total	Stage of development			Total
		†3	4	5	A		3	4	5	A		4	5	A	
Day O Control.....	1	11	24	5	262	302	26	39	90	885	1,040	50	86	91	227
Day +6 Control.....	2	0	87	19	468	574	0	26	190	728	944	60	48	147	255
Day +6 Control.....	3	0	75	139	439	653	0	30	276	729	1,035	48	70	112	230
Day +6 Control.....	4	0	8	44	392	444	0	18	260	625	903	19	72	102	193
Average No. of Worms.....		0	56·6	67·3	433	557	0	24·6	242	694	960·6	42·3	63·3	120·3	226
*CAS ANTI. 10,001 300mg/Kg	5	0	1	0	0	1	0	0	0	0	0	3	1	0	4
CAS ANTI. 10,001 300mg/Kg	6	0	0	0	0	0	0	3	0	0	3	12	8	0	20
CAS ANTI. 10,001 300mg/Kg	7	0	0	0	0	0	0	0	0	0	0	10	0	0	10
CAS ANTI. 10,001 300mg/Kg	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Average reduction.....		0	99·6%	100·0%	100·0%	99·9%	0	97·0%	100·0%	100·0%	98·8%	85·8%	96·4%	100·0%	96·2%
CAS ANTI. 10,001 450mg/Kg	9	0	0	1	0	1	0	0	0	0	0	3	0	0	3
CAS ANTI. 10,001 450mg/Kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CAS ANTI. 10,001 450mg/Kg	11	0	0	0	0	0	0	0	0	0	0	17	0	0	17
CAS ANTI. 10,001 450mg/Kg	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		0	100·0%	99·6%	100·0%	99·9%	0	100·0%	100·0%	100·0%	100·0%	88·1%	100·0%	100·0%	97·8%

* CAS ANTI. 10,001 = Cassella Anthelmintikum 10,001

† 3, 4, 5 and A = third, fourth, fifth stage and adult worms respectively

Eleven sheep had to be slaughtered on Day + 6 and facilities were not available to carry out these autopsies in the same manner as that on Sheep 1. Standard necropsies were, therefore, carried out (Reinecke *et al.*, 1963; Reinecke, 1966a, b).

The results summarized in Table 3 show that, with the exception of *O. circumcincta* in Sheep 1, worm burdens in the controls were remarkably uniform, even when compared with previous experiments (Reinecke, 1966a, b).

No worms were recovered from the colon posterior to the *ansa spiralis*.

Casella Anthelmintikum 10,001 was only 85 to 88 per cent effective against fourth stage larvae of *O. columbianum*. Against other stages of this worm and all stages of the other species present it was 96 to 100 per cent effective (Table 3).

Comment

The modified Baermann apparatus efficiently concentrated the minute larval stages of all species of worms as well as adult *T. colubriformis* in the filtrates, which represented less than 5 per cent of the mass of the ingesta. Moreover, the medium was very fine, having percolated through a sieve, and the worms were readily seen.

For a single autopsy the method was adequate but it had to be streamlined. This was achieved by using the waterbath designed by Shone & Philip (1967) omitting the illumination.

TABLE 4.—*Experimental design*

Day	No. of infective larvae dosed to each sheep		
	C. ovina	*O. circumcincta*	*G. pachyscelis*
−49	49		
−47	25		
−41	38		
−38	36		
−33	31		
−31	44		570: Sheep 15
−28	0		324: Sheep 16
−27	28		
−24	34		548: Sheep 17
−21	35		
−19	36		530: Sheep 19, 20, 21
−17	35		
−14	36		512: Sheep 22, 23, 24
−12	27	*178	
−10	35	165	
−7	36	136	
−6	33	155	
−5	34	143	
−4	35	174	
−3	164	368	
−2	164	368	
−1	164	368	
TOTAL	1,119	2,055	
0	Killed Day 0 Control: Sheep 13 Treated Sheep 17, 21, 24 with tetramisole at 15 mg/Kg intraruminally		
+1	Killed Day +1 Controls: Sheep 14, 15, 16 Killed Sheep 17, treated on Day 0		
+2	Killed Day +2 Controls: Sheep 18, 19, 20 Killed Sheep 21 treated on Day 0		
+3	Killed Day +3 Controls: Sheep 22, 23 Killed Sheep 24 treated on Day 0		

**Ostertagia circumcincta* cultures contained other larvae—see text

EXPERIMENT 2: SHONE'S WATERBATH

Materials and methods

1. *Infestation of sheep:* Twelve weaned Dorper sheep, born, reared and maintained worm-free, were infested *per os* repeatedly with infective larvae of *Chabertia ovina* (Gmelin, 1790) and *O. circumcincta*. Nine of them also received a single dose of infective larvae of *Gaigeria pachyscelis* Railliet & Henry, 1910 (see Table 4).

2. *Treatment and slaughter:* Three sheep were treated intraruminally with tetramisole at 15 mg/Kg. Details of slaughter are summarized in Table 4.

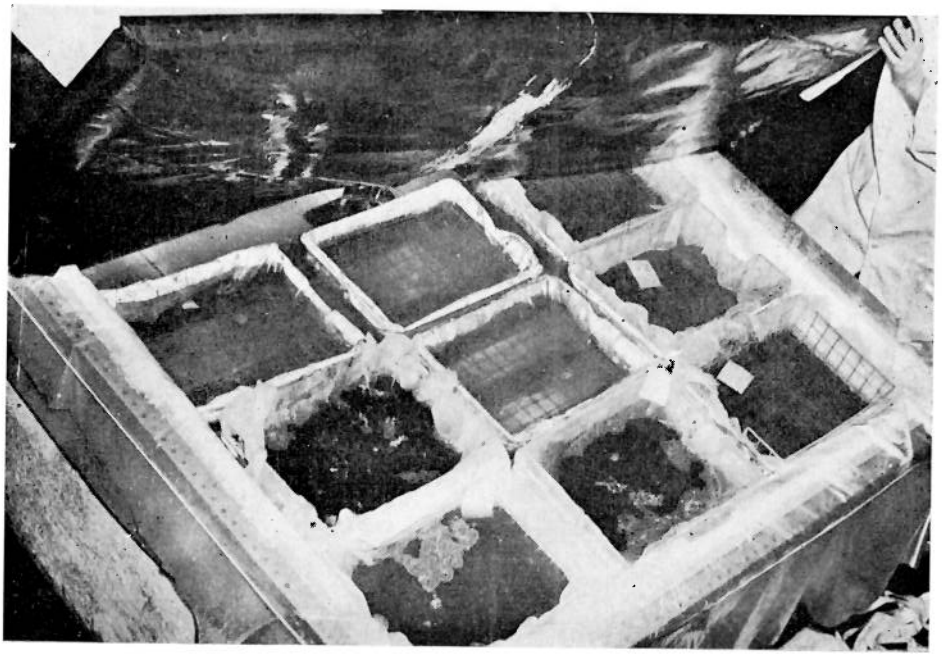

PLATE 1.—Shone's waterbath

3. *Shone's waterbath* (*see Plate* 1)*:* The inner dimensions are: length, 6 ft 6 in.; width, 4 ft 6 in. and depth 8 in. The sides are constructed of 1 in. press board extending for a distance of 1 ft 4 in. below the floor of the bath. Insulation is provided by 1 in. wide polystyrene, lining the inner sides of the pressboard for the entire 2 ft of its height. The floor of the waterbath is made of $\frac{1}{4}$ in. acrylic sheet (Perspex I.C.I., England), supported by slotted angle iron every 9 in. across the bath. It is waterproofed with a double sheet of polythene [Gunplas, G. S. Gundel (Pty.), Ltd., South Africa].

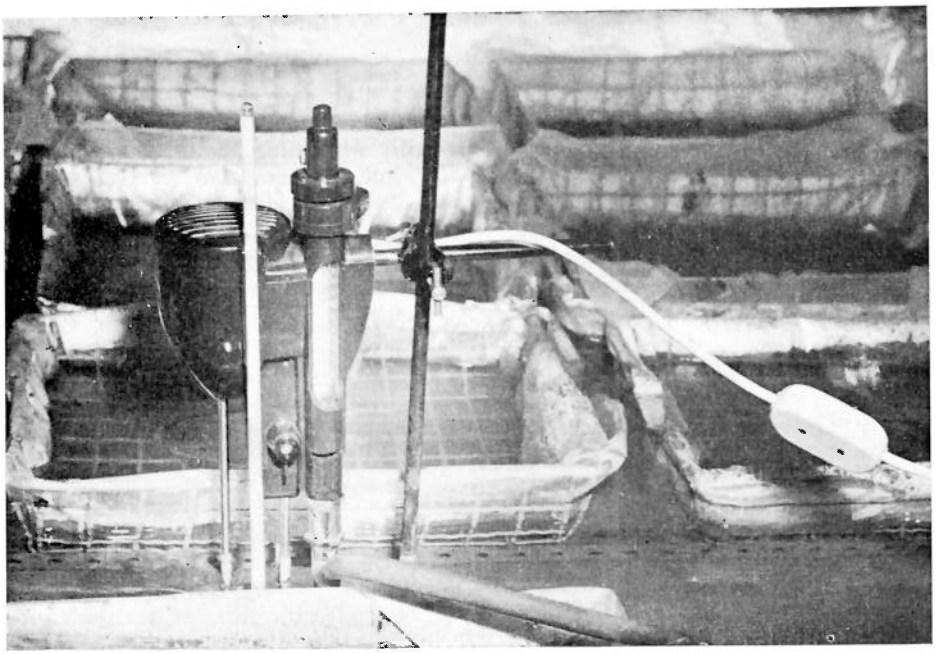

PLATE 2.—Thermomix in the waterbath

PLATE 3.—Caecal ingesta being placed on a sieve lying in a trap

The top of the bath is covered with four 15 in. wide polystyrene strips covered with black polythene sheeting (see Plate 1).

A consistent temperature is maintained in the waterbath with a Thermomix Model 2 (B. Braun, Melsungen, Germany), illustrated in Plate 2.

4. *Traps and sieves:* A frame of slotted angle iron supports 18 traps made of plastic painted, wire document trays. Each trap, which measures 15 in. × 10½ in. at the top and tapers for 4 in. to a 13½ in. × 8 in. base, is lined with a polythene sheet attached by threading masking tape through the wire mesh.

A saline solution (0·85 per cent NaCl containing 5 ml of the detergent Teepol per litre), is poured into the traps. Identical wire baskets made into sieves are then fitted into the traps, with the saline just covering their lower surfaces. The sieves used for ingesta are lined with nylon mesh (aperture 250 micron) and those used for gut wall with fibre glass gauze (aperture 3 mm). Care is taken to remove any air bubbles trapped beneath the sieves.

5. *Recovery of worms:* After opening the abdominal cavity tight double ligatures were tied an inch apart at the following points:—

(*a*) Between the omasum and reticulum and at the end of the duodenum at the duodeno-colic ligament.

(*b*) The commencement of the ileum at the origin of the ileocaecal ligament.

(*c*) The end of the *ansa spiralis*

(*d*) The rectum anterior to the anus.

After stripping the mesentery the gut was severed between the double ligatures to give four separate portions of the gastro-intestinal tract which were handled separately. The ingesta were either washed with saline into buckets and then on to the sieves or directly on to the sieves (Plate 3). The gut wall was cut into 2 cm blocks and placed on sieves.

Specimens were then left in the waterbath, as follows:—

Group	Temperature	Hours in waterbath	
		Ingesta	Gut wall
Day 0	42° C	1½–2	4–4½
Day + 1	42° C	1½–2	4–4½
Day + 2	45° C	1–2	4–5
Day + 3	45° C	1–1½	4

On the laboratory bench temperatures fell as low as 30°C before the traps were returned to the water bath. Within 10 minutes temperatures in the traps were rarely 1 to 2°C below those in the waterbath.

When specimens were removed from the waterbath worms were killed with iodine, collected and fixed as described earlier.

6. *Identification of worms:* Wherever available 60 to 80 worms were placed on slides, stained with iodine and the coverslips were sealed with Gurr's Glyceel.

Worms were identified in larval stages and moults according to the descriptions of Veglia (1915), Mönnig (1927), Ortlepp (1937), Threlkeld (1948) and Douvres (1956, 1957).

TABLE 5.—*Worms recovered post mortem from controls*

Species	Stage	Source	Sheep No. 13	14	15	16	18	19	20	22	23
O. circumcincta	*L_3	Filtrate..	52	23	0	131	55	4	8	7	0
	3M	Filtrate..	32	0	0	13	3	0	0	7	2
	L_4	Filtrate..	96	107	83	92	78	58	89	109	133
		Residue.	0	1	0	0	8	0	8	0	0
	4M	Filtrate..	9	4	9	15	4	8	22	13	7
		Residue.	0	0	0	0	0	0	4	0	0
	5	Filtrate..	76	63	78	112	116	68	94	43	99
		Residue.	0	0	4	0	12	0	11	0	0
C. ovina	L_3	Filtrate..	7	87	21	50	49	58	25	24	9
		Residue.	1	0	1	0	0	3	0	0	0
	3M	Filtrate..	0	0	0	20	0	0	4	1	2
		Residue.	1	0	0	0	0	0	0	0	0
	L_4	Filtrate..	97	247	260	319	191	233	226	137	156
		Residue.	0	3	0	0	0	1	0	0	0
	4M	Filtrate..	0	0	0	0	0	7	0	0	0
		Residue.	0	0	1	0	0	0	0	0	0
	5	Filtrate..	4	0	0	7	0	0	0	0	0
		Residue.	0	0	1	0	16	0	19	18	0
	A	Filtrate..	0	0	0	1	0	0	0	0	0
		Residue.	0	0	0	17	38	0	0	0	0
G. pachyscelis	L_4	Filtrate..	Not infested	Not infested	0	0	Not infested	0	129	30	207
		Residue.			0	4		0	75	0	0
	5	Filtrate..	Not infested	Not infested	0	7	Not infested	0	0	0	0
		Residue.			17	153		0	0	0	0
T. colubriformis	L_3	Filtrate..	12	40	8	14	0	17	12	4	3
	3M	Filtrate..	0	4	0	0	0	0	0	0	0
	L_4	Filtrate..	12	50	25	23	50	72	41	269	53
	4M	Filtrate..	0	0	4	0	0	0	0	0	0
	5	Filtrate..	8	38	17	16	41	13	39	48	34
	A	Filtrate..	0	0	0	0	0	13	15	18	0

* L = Larvae M = Moult A = Adult

Results

The presence of *T. colubriformis* in reasonable numbers (32 to 339) and of *Haemonchus contortus* (Rudolphi, 1803) in moderate numbers (7 to 27) indicated that the cultures dosed to sheep were contaminated with these species. The sheep were experimentally infested with *O. circumcincta*, *C. ovina* and *G. pachyscelis* (Table 4). It is more than likely that the cultures of *O. circumcincta* were contaminated, as infective larvae of *T. colubriformis* closely resemble this species, and were probably incorrectly diagnosed.

The filtrate contained almost all the smaller larval stages of all species, the fifth stage of *O. circumcincta* and all stages of *T. colubriformis* (Table 5), whereas the fifth stage and adult worms of the larger species, i.e. *C. ovina*, *G. pachyscelis* and *H. contortus*, remained in the residue.

It was encouraging to note that the majority of worms in the gut wall migrate through the sieves into the filtrate. Exceptions, recovered from digested gut, are summarized below:—

Sheep No.	Stage	No. of worms	Species
18	Fourth	8	*O. circumcincta*
20	Fourth moult	4	*O. circumcincta*
15, 17 & 20	Fifth	3, 12 & 11	*O. circumcincta*
19	Third	3	*C. ovina*
20	Fourth	4	*H. contortus*

It did not seem worthwhile to digest the gut to release these moderate numbers.

The examination for *C. ovina* showed that it was absent posterior to the *ansa spiralis*. Worms in the fourth moult were, however, recovered from the abomasal filtrate (Sheep 19) and 19 fifth stage specimens from the jejunal residue. Their presence, anterior to the ileum in these advanced stages of development, is surprising.

The efficacy of tetramisole against fourth stage larvae, fourth moult and fifth stage *O. circumcincta* was poor, falling as low as 48 per cent, 67 per cent and 71 per cent respectively. Elsewhere it was from 99 to 100 per cent effective.

Comment

This technique of collecting worms represents a major advance. It is not only highly efficient for collecting larval stages from ingesta but the histotrophic phases migrated into the saline filtrates in large numbers.

Little advantage seemed to be gained by boosting the temperature to 45°C as results at 42°C were equally satisfactory.

It was important to establish the efficiency of this method for collecting *O. columbianum* from the gut wall.

EXPERIMENT 3: RECOVERY OF *O. columbianum* FROM THE GUT WALL

Four weaned Dorpers, born, reared and maintained worm-free, were infested orally with infective larvae of *O. columbianum* as follows:—

Day	Sheep 25	Sheep 26	Sheep 27	Sheep 28
−6	0	0	2,000	3,000
−5	0	4,000	2,000	3,000
−4	0	4,000	0	0
−3	7,800	0	4,000	6,000
−2	5,140	8,000	0	0
−1	5,800	0	0	0
TOTAL	18,740	16,000	8,000	12,000

Sheep were killed on Day 0 and the intestines from the pylorus to the end of the *ansa spiralis* removed.

In Sheep 25 the ingesta from the small intestine, caecum and colon were placed on separate sieves. Saline in the traps was at a temperature of 37·5°C initially but fell to 25°C because they were left on the laboratory bench for an hour. The sieves were transferred to saline in traps and incubated for a further five hours in the waterbath at 40°C.

A total of 298 worms was recovered from the filtrates in the first hour and only 21 after a further five hours in the waterbath. In subsequent autopsies the ingesta were discarded after opening the gut.

TABLE 6.—O. columbianum *recovered from the intestinal wall*

Time in hours	Sheep 25		Sheep 26		Sheep 27		Sheep 28	
	Wall	M.S.*	Filtrate	Digesta	Filtrate	Digesta	Filtrate	Digesta
0·5	—	—	—	—	—	—	—	763
1·0	305	248	469	322	—	—	—	—
1·5	—	—	—	—	—	292	—	—
2·0	—	—	209	455	—	—	—	—
2·5	—	—	—	—	—	383	—	—
3·0	193	346	192	733	—	—	—	—
3·5	—	—	—	—	—	—	—	—
4·0	—	—	—	—	327	—	395	—
5·0	—	—	445	—	—	—	—	—
6·0	73	310	—	—	—	—	—	—
7·0	—	—	235	—	—	—	—	—
TOTAL	571	904	1,550	1,510	327	675	395	763
Digestion of Residue	38	61	341	—	78	—	95	—
GRAND TOTAL	609	965	1,891	1,510	405	675	490	763

* Mucosa and Serosa

For comparative purposes the gut wall was divided into two equal parts by weight. In Sheep 25 the two halves of each part of the intestinal tract, viz. the small intestine, caecum, and colon, were kept separate giving six specimens. In other autopsies they were pooled to form two specimens (Sheep 26, 27 and 28).

Worms recovered from the filtrates are given in Table 6. The first column indicates the total number of hours spent in the waterbath at 40°C because in most cases, sieves were transferred at different intervals to freshly prepared traps.

The wall was macerated as follows:—

Sheep 25

One-half, referred to as *wall* in Table 6, was cut in pieces.

The mucosa and muscular layers, referred to as *mucosa*, of the other half were stripped off the *serosa* by scraping with a blunt spatula. The mucosa and serosa from the small intestine were kept separate and placed on different sieves while those from the caecum and colon were pooled. After an hour in the waterbath the sieves were placed in freshly prepared saline in new traps. Two hours later this procedure was repeated and the sieves were removed after a further three hours. These are the periods referred to as one, three and six hours in Table 6.

The residues were stored at 4°C overnight and then digested for 7 hours in pepsin in an incubator at 45°C the following day.

The worms were killed with iodine, fixed and collected as previously described.

Fewer worms were recovered from the wall than from the mucosa and serosa; the mucosa of the small intestine yielded 641 worms and its serosa only 21 worms.

At subsequent autopsies, therefore, all intestines were scraped. The mucosa was retained but the worms on the serosa were merely rinsed off and pooled with the respective mucosal scrapings and the serosa was then discarded.

Sheep 26

Each half of the mucosa was placed in a blender (Ato-mix, M.S.E. England) for 10 seconds at half speed to produce a thick homogenate. The half placed in saline is known as *filtrate* and the other half, in pepsin/HCl, as *digesta* (Table 6).

Sieves were transferred to freshly prepared traps every hour for three hours, by which time the mucosal homogenate in pepsin/HCl was completely digested. The filtrate was left for two further two-hour periods. The residue, after storage overnight at 4°C, was placed in pepsin/HCl in a trap at 40°C and completely digested in $1\frac{1}{4}$ hours.

Equivalent numbers were recovered after seven hours filtration (1,550) and three hours digestion (1,510). Digestion of the filtrate residue yielded a further 341 larvae (Table 6).

The cuticle started to separate from some of the larvae two hours after the commencement of digestion and 80 per cent of the worms collected from the three hour digesta were abnormal morphologically. Those in the filtrate remained normal even after seven hours in saline.

Homogenization had, therefore, increased the yield of worms. Subsequently the mucosa was placed in the blender for 30 seconds at half speed to form a very fine homogenate.

Sheep 27

One half was placed in saline for four hours and the residue digested for a further hour; this yielded 405 worms. Immediate pepsin digestion for an initial period of an hour-and-a-half followed by another hour was more successful, 675 worms being recovered (Table 6). Moreover the morphology of the larvae was little affected by $1\frac{1}{2}$ hours in pepsin/HCl.

It was desirable to compare trypsin with saline extraction in the last necropsy of this series.

Sheep 28

Immediate digestion of the one-half with trypsin was completed in 30 minutes and yielded 763 worms (Table 6). The mucosa of the other half was placed in saline for four hours and its residue transferred to freshly prepared trypsin (5 gm trypsin + 5 gm $NaHCO_3$ per litre of water, adjusted to a pH of 7 to 8). It was incompletely digested after two hours and only 490 worms were recovered from this half. The differences in these results cannot be explained.

The trypsin and $NaHCO_3$ were from the same bottles, the solutions were freshly prepared and the pH adjusted to lie between 7 and 8. Prior to digestion the filtrate had spent four hours in saline, i.e. 0·85 per cent NaCl + 5 ml Teepol per litre. What effect, if any, this had on digestion is not clear.

Comment

The larval stages of this parasite in the gut wall have always been difficult to collect. The histotrophic stages of *C. ovina* and *O. circumcincta* readily migrate from the gut wall through the sieves into the filtrate. The same stages of *O. columbianum* have, however, proved more refractile and migration into saline filtrates was not yet completed even after seven hours in the waterbath. This means that enzyme digestion is still the method of choice if all the worms present are to be collected.

Discussion

The original Baermann Apparatus was used to extract larvae from soil (Baermann, 1917). The author used funnels 25 cm in width at the top, closed at the bottom with a clamped rubber tube and supported in a stand. A circular piece of wire gauze is allowed to seat itself about 5 cm from the upper surface and is covered with mutton cloth. Soil containing larvae is spread over the cloth to a depth not exceeding 2 cm and warm water (40°C) poured down the edge of the funnel until it just covers the surface of the soil. This is left overnight, a few ml are drawn off by opening the clamp the next morning and the worms found in the sediment. In the author's experience this apparatus is not very efficient for the collection of free-living stages of parasitic nematodes (Reinecke, 1960).

Modifications of this technique were successfully used in this laboratory to extract all stages of development of *Dictyocaulus filaria* (Rudolphi, 1809) from lymph glands, lungs, etc., by P. J. S. Anderson, Johannesburg (1966 in a personal communication). Initial attempts to extract worms with sieves in plastic trays in incubators were clumsy (Sheep 1). The development by D. K. Shone, Johannesburg (1966 in a personal communication) of a large waterbath streamlined these procedures and this method has been tested and the results reported above.

The following points must be emphasized if the best results are to be obtained:—

1. *Live worms:* Necropsies must be carried out as rapidly as possible and all specimens placed in the waterbath within three hours of slaughter of the sheep. This is essential as parasitic nematodes start dying within a few hours of slaughter of the host. Tne entire success of this system is dependent on the worm wriggling through a hole in the sieve into the filtrate.

2. *Removal of trapped air bubbles:* Worms cannot break through the water into air and then back into fluid. All air trapped below the sieves must therefore be removed (S. P. Kruger, Onderstepoort, 1966 in a personal communication).

3. *Thickness of ingesta or gut wall:* The worms are required to creep out of their habitat through a sieve into the saline in the trap. The thinner the medium is spread on the surface of the sieve, the less it obstructs the downward migration of the worms. This is the main reason for the use of flat document trays to support the nylon gauze which acts as a sieve. Ingesta or gut wall placed on the surface of the gauze are spread thinly over a large area and are never allowed to exceed 5 mm in thickness. If more material is present, e.g. caecal and colonic ingesta, an extra sieve is used.

4. *Depth of traps:* Worms collected in filtrates rarely exceed 2 cm in length. It is therefore only necessary to lift the sieve 2·5 to 3 cm from the floor of the trap to allow worms to fall freely into the filtrate.

5. *Temperature:* In the traps temperatures rarely fall more than one or two degrees below the temperature of the surrounding water in the bath. It is therefore kept at 40° C to enable the worms to be as near as possible to normal temperatures. This may not be critical for ingesta since most worms migrate into the trap even if trays are left on the laboratory bench at 25°C for an hour (Sheep 25). In the histotrophic phases of *O. columbianum*, however, this may be more important as many larvae are still left in the gut wall seven hours after being placed in traps containing saline, although the temperature in the waterbath was kept at 40°C throughout (Sheep 26).

6. *Fluid in traps:* Worms migrate from dry into moist surroundings (Kruger, 1966, personal communication). In an attempt to encourage this migration, a minimum amount of saline is used to cover the sieve (1 to 2 mm).

7. *Waterproofing of traps:* In these experiments thin polythene sheeting was used, and is still being used where enzymes are essential for the digestion of gut wall. Recently, however, galvanised iron traps 1/16 in. thick have replaced polythene lined traps where filtrates of ingesta and the gut wall are being collected. These metal traps are better conductors of heat than polythene sheeting.

8. *Histotrophic phases:* With one notable exception histotrophic phases can be collected from saline filtrates after four hours in the waterbath. Even after seven hours some *O. columbianum* remain in homogenates of the mucosa (Sheep 26). In this species digestion in plastic trays with trypsin for 30 minutes (Sheep 28) or pepsin for $2\frac{1}{2}$ hours is very efficient (Sheep 27). Pepsin, however, is not ideal because it begins to digest the worms themselves, within two hours.

9. *Fifth stage and adult worms:* In the larger species, e.g. *C. ovina*, *O. columbianum*, *H. contortus* and *G. pachyscelis*, most fifth stage and adult worms do not migrate through the sieves (of 250 micron aperture). This is not a major disadvantage

because they can easily be detected macroscopically. With the exception of *Cooperia* spp. which become entangled in the mesh (Shone, 1967, personal communication), all smaller species migrate into the filtrates.

When these principles are observed the methods described in this paper are the best the author has used for the recovery of larval stages post mortem, concentrated in as little ingesta as possible.

This waterbath is now a permanent feature of the laboratory and the expense of the stainless steel bath at present in use is justified.

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References

BAERMANN, G., 1917. Eine einfache Methode zur Auffindung von Ankylostomum—(Nematoden)—Larven in Erdproben. *Geneesk. Tijdschr. Ned-Indië*, 57, 131–137.

DOUVRES, F. W., 1956. Morphogenesis of the parasitic stages of *Ostertagia ostertagi*, a nematode parasite of ruminants. *J. Parasit.*, 42, 626–633.

DOUVRES, F. W., 1957. The morphogenesis of the parasitic stages of *Trichostrongylus axei* and *Trichostrongylus colubriformis* nematode parasites of cattle. *Proc. helminth. Soc. Wash.*, 24, 4–14.

MÖNNIG, H. O., 1927. The life-histories of *Trichostrongylus instabilis* and *T. rugatus* of sheep in South Africa. *Rep. vet. Res. Un. S. Afr.*, 11/12, 229–251.

ORTLEPP, R. J., 1937. Observations on the morphology and life-history of *Gaigeria pachyscelis* Raill. and Henry, 1910: a hookworm parasite of sheep and goats. *Onderstepoort J. vet. Sci. Anim. Ind.*, 8, 183–212.

REINECKE, R. K., 1960. A field study of some nematode parasites of bovines in a semi-arid area with special reference to their biology and possible methods of prophylaxis. *Onderstepoort J. vet. Res.*, 28, 365–464.

REINECKE, R. K., 1966a. A larval anthelmintic test. *Jl S. Afr. vet. med. Ass.*, 37, 27–31.

REINECKE, R. K., 1966b. The value of uniform worm burdens in the larval anthelmintic test. *Jl S. Afr. vet. med. Ass.*, 37, 133–142.

REINECKE, R. K., HORAK, I. G. & SNIJDERS, A. J., 1963. Techniques for testing anthelmintics against immature *Oesophagostomum columbianum*. *Proc. Int. Conf. Wld Ass. Adv. vet. Parasit.*, 1, Hanover, 1963. The evaluation of anthelmintics. 167–180.

SHONE, D. K. & PHILIP, J. R., 1967. Anthelmintic and toxicity studies with tetramisole. 1 Anthelmintic efficacy. *Jl S. Afr. vet. med. Ass.*, 38, 165–176

THRELKELD, W. L., 1948. The life history and pathogenicity of *Chabertia ovina*. *Tech. Bull. Va agric. Exp. Stn*, 3.

VEGLIA, F., 1915. The anatomy and life history of the *Haemonchus contortus* (Rud.). *Rep. vet. Res. Un. S. Afr.*, 3/4, 347–500.