# AN ANTHELMINTIC TEST FOR LARVAL STAGES OF SHEEP NEMATODES

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## INTRODUCTION

In recent years marked progress has been made in the efficacy of new anthelmintics. Many thousands of these compounds are synthesized by the pharmaceutical industries and the primary and secondary laboratory animal screens, through which they pass initially, have been carefully described and reviewed in the literature (Standen, 1963; Baker, 1963). After undergoing these tests promising compounds are subjected to clinical and field trials in domestic animals.

Standard tests for the older anthelmintics, which were only effective against the adult worms, were accurately described by Gordon (1950). They comprise the *critical test* of Hall & Foster (1918), the *controlled test* of Moskey & Harwood (1941) and a test based on *faecal worm egg counts*. These methods have been thoroughly examined and modified, and their defects have been described, by Reinecke, Snijders & Horak, 1962; Reinecke & Rossiter, 1962; Reinecke, 1963.

The efficacy of modern anthelmintics against all parasitic stages has necessitated the development of new methods for determining their degrees of efficiency. The first was described by Banks & Michel (1960), who administered a single massive dose of larvae of one species and treated selected animals on specific days after infestation. Subsequently this method was refined by Gibson (1964), who tested an anthelmintic either against the parasitic third stage, fourth stage or adult worm. The larval anthelmintic test which has been evolved at this laboratory is designed to determine the effect of the compounds against all the parasitic developmental stages (Reinecke, 1963, 1966a, b, 1967; Reinecke *et al.*, 1962; Reinecke, Horak & Snijders, 1963; Reinecke & Anderson, 1967).

The object of this paper is to describe this test.

## BASIC REQUIREMENTS

The prerequisites for the larval anthelmintic test are adequate animal housing, worm-free lambs and pure nematode strains.

*Worm-free lambs:* It is essential to use fully susceptible hosts. The more uniform the worm burdens are in experimentally infested sheep, the more reliable is the interpretation of results, when comparing the worms remaining in treated sheep with those in untreated controls. For this purpose worm-free lambs have proved to be the only suitable hosts for experimental infestations (Reinecke, 1966a, b; Reinecke *et al.*, 1963).

Dorpers (Dorset Horn × Black Head Persian), unlike Merinos, are rapidly maturing, fertile animals and the ewes are good mothers. They are allowed to lamb in pens with concrete floors, which are cleaned regularly with brooms and a strong stream of water. Lucerne hay *ad lib.*, green feed and concentrates are fed in troughs. In addition the lambs are creep fed with a ration containing 16 per cent protein.

The lambs grow rapidly and to prevent white muscle disease it is necessary to dose them orally at four weeks and again at weaning with 3 mg of selenium in the form of sodium selenite.

Despite these precautions a few specimens of *Cysticercus tenuicollis, Skrjabinema ovis* (Skrjabin, 1915) and *Trichuris parvispiculum* Ortlepp, 1937 have been recovered from some of the lambs but the majority remain worm-free.

*Pure nematode strains:* Pure strains of the following species are maintained:—

*Haemonchus contortus* (Rudolphi, 1803)

*Trichostrongylus colubriformis* (Giles, 1892)

*Nematodirus spathiger* (Railliet, 1896)

*Chabertia ovina* (Fabricius, 1788)

*Oesophagostomum columbianum* (Curtice, 1890)

*Gaigeria pachyscelis* Railliet & Henry, 1910

A strain of *Ostertagia circumcincta* (Stadelmann, 1894) which was also maintained was lost recently and has been replaced with a mixture of *O. circumcincta* and *O. trifurcata* Ransom, 1907.

It is essential that large numbers of infective larvae of all these species are freely available.

To keep these strains pure and prevent cross-infestations each species is established in wethers housed in separate pens each of which is provided with water, feeding troughs and brooms for cleaning purposes.

Two or three wetners are infested with each species. Checks are made on the degree of infestation of each sheep by faecal egg counts and on the purity of the strains by the examination of infective larvae from cultures. They are examined weekly and any loss of infestation or contamination with another species results in the immediate removal of the specific " donor " sheep.

Adequate care must be taken to check the purity of any strain, particularly in the case of species which may resemble each other morphologically, e.g. *O. circumcincta* and *T. colubriformis*. The incorrect identification of infective larvae has been the cause of cross-infestation in at least two anthelmintic tests (Reinecke, 1967; Reinecke & Anderson, 1967).

*Larval Anthelmintic Test*

Before this test is carried out data proving the efficacy of the compound against a wide range of adult nematodes must be provided. If these data are satisfactory tests can be planned along the following lines.

<center>MATERIALS AND METHODS</center>

## 1. *Experimental hosts*

It has been shown that a controlled test is essential and worm-free weaned sheep are the most suitable experimental animals. Since they are extremely expensive to rear the greatest economy must be exercised in their use. Numerous experiments have been carried out to try and achieve uniform worm burdens in experimentally infested sheep. This would increase the reliability in the interpretation of results and decrease the number of controls to a minimum of four sheep (Reinecke *et al.*, 1963; Reinecke, 1963, 1966a, b).

At least four, preferably five, sheep should be treated. This will indicate the percentage reduction in the number of parasites, or " Intensive Effect " in a group of animals, but not the proportion of animals successfully treated, or " Extensive Effect ", of the anthelmintic (Boray, 1963). Larger numbers of animals will have to be treated to indicate the latter and this will only be justified if the compounds were sufficiently effective to warrant the extra cost.

## 2. *Period of experimental infestation*

Larvae have been dosed repeatedly throughout the prepatent period in most of the tests carried out (Reinecke, 1963, 1966a, b, 1967; Reinecke *et al.*, 1963). Recently the method was refined by infesting the sheep in three different ways.

Some groups were infested every day for three days, to cover the third stage larvae and the third moult. Other groups were infested daily from four to twelve days before treatment to cover the fourth stage, fourth moult and fifth stage of such species as *H. contortus*, *O. circumcincta*, *T. colubriformis* and *N. spathiger*. Further groups were infested on seven separate occasions from 12 to 26 days prior to treatment to include the late fourth to early fifth stage of *O. columbianum* and *C. ovina*.

The merits of this refinement will be dealt with later when the slaughter of controls is discussed.

*Gaigeria pachyscelis* has not lent itself to trials of this nature. Infective larvae infest sheep percutaneously and only the first dose has established itself despite several attempts at repeated infestation (Reinecke, 1966a, b). Shone & Philip (1967) and Anderson (unpublished observations) have also been unsuccessful in attempts at reinfestation. It is therefore necessary with this species to carry out a modified Banks & Michel (1960) test. One dose of infective larvae is placed on the skin and the anthelmintic is administered to selected sheep during the prepatent period. Their residual worm burdens are compared with undosed controls killed some time later (Reinecke, 1966a, b).

## 3. *Preparation of larval doses*

McMaster faecal collecting bags are attached overnight and the faeces collected. They are mixed with vermiculite and cultures made using a modified version of the methods described by Roberts & O'Sullivan (1950).

The mixture is placed in a wide-mouthed glass fruit jar of approximately 1 litre capacity to a depth of 5 cm and tamped down with the blunt end of a test tube. After incubation for 7 days at 26° C any larvae, that have migrated up the inner surface of the jar, are washed down on to the surface of the culture. After exposure to

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indirect light for 1 hour only those live larvae which have subsequently migrated upwards are washed into 100 ml measuring cylinders with the aid of a plastic wash bottle.

Cultures of *N. spathiger*, however, are prepared by using a method described by Horak (personal communication). Eggs are separated from faeces as follows:—

(i) Infested faeces are emulsified with water and washed into a bucket through a 100 mesh to the linear inch sieve (150 micron apertures). The residue trapped on the sieve is discarded.

(ii) The eggs are concentrated by washing the filtrate in the bucket through a 400 mesh to the linear inch sieve (37 micron apertures) trapping the eggs on the surface of the sieve.

(iii) Further concentration of eggs is obtained by washing the residue off the sieve, centrifuging it, discarding the supernatant, mixing the sediment with 40 per cent sugar solution and repeating the centrifugation to float the eggs into the supernatant.

(iv) The sugar solution is removed by washing the supernatant through a 400 mesh sieve.

(v) The residue is washed off the sieve into a wide mouthed jar of 1 litre capacity which is then filled with water. The jar is left for three minutes allowing most of the eggs to settle in the sediment. The supernatant is poured onto a 400-mesh sieve and the process repeated five to ten times depending upon the faecal debris present, to separate the eggs from the faeces.

Eggs freed of faeces are incubated in petri dishes in water for three weeks at 27°C by which time the infective larvae hatch.

Thereafter, with all species the larval suspension is brought up to a volume of 100 ml by the addition of water and thoroughly mixed by blowing air through it with a pipette. Four to six aliquots are withdrawn, the larvae killed with Lugol's iodine, counted and the total number estimated. Simultaneously further larvae are withdrawn, killed with iodine and at least 200 identified to check the purity of the culture.

The volume of suspension which contains the desired number of larvae is withdrawn and pipetted onto a small piece of blotting paper 3 cm in diameter, placed in a Buchner funnel and the excess water removed with the aid of a vacuum flask connected to a negative pressure water pump. The blotting paper is removed, folded and fitted into a No. 60-gelatine capsule (Parke-Davis). This procedure is repeated until at least one dose in excess of the number required to infest the sheep has been prepared.

During this process two doses are withdrawn at random and the larvae counted separately to serve as a cross check of the actual number dosed. Each sheep is dosed *per os* with the aid of a mouth gag at such intervals as are required for the experiments. The excess dose is returned to the laboratory, the larvae washed off the blotting paper and checked for motility.

In the case of *G. pachyscelis* the number of infective larvae required to infest the sheep is withdrawn and pipetted into a centrifuge tube. This process is repeated until a number of doses in excess of those required has been pipetted into separate

tubes. The actual number withdrawn is checked in one or two tubes. An area 3 to 5 cm in diameter is clipped on the sheep's back between the shoulder blades, the area washed with water and the larval suspension placed directly onto the skin.

It is desirable that the entire dose be concentrated in 1 or 2 ml as the suspension is easily lost, particularly if the sheep has a thick coating of lanolin on the skin.

### 4. *Number of infective larvae dosed*

*Gaigeria pachyscelis*: These worms are virulent, 100 or more worms in the intestine killing sheep within 100 days of infestation, and if they are permitted to develop to patency at 70 days only 200 infective larvae should be placed on the skin. In experiments where the sheep are to be killed within five weeks of infestation 500 larvae are not excessive. The other species are dosed *per os* at regular intervals throughout the prepatent period, up to and including the fourth moult.

*Oesophagostomum columbianum* and *Chabertia ovina*: These species are dosed every second day for 18 days and then daily for the last eight days before treatment. Forty to fifty larvae per dose, with a total of 600 to 800 larvae for the entire period, have resulted in uniform worm burdens of both species (Reinecke, 1967; Reinecke & Anderson, 1967).

In the following four species the total period of dosage with larvae need not exceed 12 days, by which time the first larvae dosed will have reached the early fifth stage. The following numbers of infective larvae are recommended.

*Haemonchus contortus* and *Trichostrongylus colubriformis:* A daily dose of 250 to 400 larvae, with a total of not more than 5,000, has given the most satisfactory results for both species (Reinecke, 1966b).

*Ostertagia circumcincta:* Daily doses of 100 to 250 larvae, up to a maximum of 1,500 to 3,000, have given the best results (Reinecke, 1967).

*Nematodirus spathiger:* Doses of 300 larvae every second day, with a total of 2,000, give better results than 600 larvae per dose up to a maximum of 4,000.

### 5. *Mixed infestations*

Gibson (1964) stated that mono-specific infestations in artificially infested animals are the best for use in the controlled test. He used 12 lambs to test an anthelmintic against a single species. This is highly desirable but does not permit the use of a wide range of parasites unless large numbers of worm-free sheep are available, which is rarely the case and mixed infestations have to be used.

Reinecke (1966a, b; 1967) established the following species concurrently in uniform number in the same lambs:—

    (i) *H. contortus, T. colubriformis, O. columbianum* and *G. pachyscelis;*

    (ii) *O. circumcincta, C. ovina* and *G. pachyscelis;*

    (iii) *O. circumcincta, T. colubriformis* and *O. columbianum.*

Anderson (personal communication) was able to infest sheep with remarkably uniform worm burdens of *H. contortus* and *N. spathiger* but there is experimental evidence to show that *T. colubriformis* reacts with *N. spathiger* to the detriment of both species (C. Sussana Müller, Onderstepoort, unpublished observations). This does not confirm observations by Kates & Turner (1953), who established both species concurrently in large numbers.

It is known that the presence of *O. circumcincta* influences the establishment of *H. contortus* (Turner, Kates & Wilson, 1962; Reinecke, 1966b). Therefore it is undesirable to mix these two species, or *T. colubriformis* and *N. spathiger*, in the same host.

## 6. *Treatment*

The sheep have been infested artificially so that by a certain day, known as Day 0, they have worms in all stages of development. On Day 0 treatment of one or more groups of sheep takes place. Drugs are dosed intraruminally for ease of administration, to avoid wastage and to prevent inadvertent stimulus of the oesophageal-groove reflex, which may complicate the interpretation of results.

Manufacturers of drugs invariably underestimate the dose required. In determining the efficacy of a compound it is essential therefore to increase the dosage by 50 or even 100 per cent (Reinecke 1966a, b; 1967; Reinecke *et al.*, 1963).

## 7. *Slaughter*

(*a*) *Controls:* On the day of treatment (Day 0) it is essential that a control be slaughtered to ascertain the precise state of development of the larvae at that time. It has been stated that the Day 0 control indicates the number of worms in various larval stages on the day of treatment (Reinecke, 1963, 1966a). This statement does not apply to all the larval stages. Many of those larvae which have been in the host for less than three days at slaughter will either be missed on microscopic examination or will not be recovered because they have not yet reached their final habitat (Reinecke *et al.*, 1963). They should therefore be designated as one-day to three-day old worms and merely regarded as indicators of larval viability (Reinecke & Anderson, 1967). Controls killed two or three days later invariably yield larger numbers of worms at autopsy.

Once the worms are in the fourth and fifth stage, however, both the number of each stage and the proportion between them on Day 0 are very similar to the position two or even three days later. Thus worms in these stages in controls, killed on Day 0 or Day + 1, can be compared with those in treated sheep killed on Day + 3 (Reinecke & Anderson, 1967).

(*b*) *Treated sheep*: With an anthelmintic such as methyridine which acts very rapidly, sheep can be killed within one or two days of treatment (Reinecke, 1963; Reinecke *et al.*, 1962). It is, however, only reasonable to allow those larvae in the histotrophic phase which have been killed by the anthelmintic to be expelled and this may take longer. For example the anthelmintic effect of both thiabendazole and methyridine against *O. circumcincta* shows a marked improvement 72 hours after treatment compared with the results in sheep killed 24 hours after treatment (Reinecke, 1963).

## 8. *Autopsy*

Worms tend to localise themselves in strictly defined areas. It is a great help if, prior to recovery of the worms and microscopic examination, the gastro-intestinal tract is divided at autopsy into the various parts which the different species normally

inhabit. This is best done immediately after slaughter and opening the abdominal cavity. Tight double ligatures tied at the following points will separate almost all the worms mentioned in the list below:—

*H. contortus* and *Ostertagia* spp.: Between the omasum and reticulum (because there is a convenient notch there, not present at the fundus) and the duodenum at the duodeno-colic ligament:

*T. colubriformis* and *N. spathiger:* The pylorus and the jejunum 7m from the pylorus:

*O. columbianum* and *C. ovina:* The commencement of the ileum at the ileo-caecal ligament and the end of the *ansa spiralis.* In the latter case landmarks are the tip of the caecum and the last coil of the *ansa spiralis;* the ligatures are placed where an imaginary line joining these structures crosses the descending colon. These species have very rarely been recovered from the rest of the colon or rectum and it is therefore discarded.

The mesentery is stripped and the gut cut between the double ligatures to release the various portions of the gastro-intestinal tract. Each portion is thereafter dealt with separately.

## 9. *Recovery and concentration of worms*

Worms are concentrated in a minimal amount of ingesta or digested gut in a modified Baermann apparatus placed in Shone's waterbath (Shone & Philip, 1967; Reinecke, 1967). The sieve, which consists of nylon mesh (250 micron apertures) supported by a plastic painted wire document tray, is placed in a waterproof trap of similar size to seat itself 2 to 3 cm above the trap's floor. The trap contains saline and teepol (5 ml/litre), and is either a document tray lined with plastic sheeting or is made of galvanized iron, or stainless steel.

Ingesta or homogenated gut wall is placed on the sieve in the trap and incubated at 40° C in the waterbath. Worms migrate through the nylon mesh into the filtrate within an hour in the case of ingesta and from the gut wall within three hours. To release *O. columbianum* from the intestinal wall the same procedure is followed except that the gut wall is digested with 1 per cent pepsin plus 3 per cent concentrated HCl. It is essential when digesting gut to remember that the traps and sieves must be made of chemically inert materials, such as plastic or stainless steel, to avoid interfering with the digestive reaction. In the waterbath at 40°C homogenated gut is completely digested within 3 hours (Reinecke, 1967).

Traps are removed from the waterbath and the filtrate in the trap and the residue on the nylon mesh placed in separate buckets. The nylon mesh is examined macroscopically for adult worms which may be trapped there. Worms are either killed by placing the buckets in boiling water, stirring the contents vigorously until the temperature reaches 60°C, or by the addition of 3 ml of concentrated iodine per litre of suspension. The suspensions are washed through 400-mesh sieves and the ingesta and worms trapped on the sieves' surface are washed into glass jars and preserved with an equal quantity of 10 per cent formalin.

All stages of *T. colubriformis* and *O. circumcincta,* migrate into the filtrate, while only the fifth stage and adults of *O. columbianum, C. ovina, G. pachyscelis* and *H. contortus* remain in the residue (Reinecke, 1967).

This method was recently tested on 23 autopsies of sheep infested with *N. spathiger*. Although a mean of only 8·4 per cent of developmental stages remained in the residue, in two autopsies it rose as high as 26·4 and 30·0 per cent respectively.

The minute larval stages are concentrated in the filtrate which represents less than 5 per cent of the bulk of the ingesta. They are counted with the aid of a stereomicroscope.

The presence of larval stages in the residue is checked by examining a one tenth aliquot microscopically. If present the balance of the residue is examined as described below, if absent, the balance is examined macroscopically for adult worms.

## 10. *Counting the worms*

Water is added to each separate specimen to bring the quantity to 1 litre. If the ingesta is bulky water can be added to 2 or 3 litres. Cylindrical glass specimen jars 10 cm in diameter $\times$ 30 cm in depth are excellent for this purpose. The suspension is thoroughly mixed by means of a strong stream of air blown through a glass tube 1 cm in diameter, connected by a rubber tube to an air pump. Simultaneously the suspension is vigorously stirred. A 20 ml glass pipette with the tip cut off to increase the diameter of the bore, is used to withdraw a volume of fluid which is then transferred to calibrated glass measuring cylinders of either 100 or 300 ml capacity. The procedure is repeated until a volume of not less than one tenth, more often one fifth, has been collected from each pool. Three such aliquots are collected from each pool.

This dilution technique is remarkably accurate for estimating large worm burdens. Where worm burdens are lower than 600, however, it is unreliable and total counts are carried out.

Each aliquot is stained with 1 to 2 ml of concentrated iodine solution until it assumes the colour of port wine. This facilitates the detection of the minute larvae which stain a darker colour than the surrounding ingesta. A few ml are poured into a square perspex counting chamber ($10 \times 10 \times 1$ cm), which has parallel lines, 5 mm apart, scored across its upper surface. The worms are counted with the aid of a stereoscopic microscope. If available, 100 worms are collected at random with dissecting needles or micropipettes and transferred to a small labelled plastic specimen bottle. This contains formalin, picric acid and triethanolamine which decolourize the worms (Reinecke, 1967). They are subsequently transferred to glass slides, a drop of iodine added, the coverslips sealed with Glyceel and the worms identified with the aid of a standard microscope.

Larval stages are identified according to the description given by the following authors:—

| Species | Authors |
|---|---|
| *Haemonchus contortus* | Veglia, 1915 |
| *Oesophagostomum columbianum* | Veglia, 1923 |
| *Trichostrongylus colubriformis* | Mönnig, 1927; Douvres, 1957 |
| *Gaigeria pachyscelis* | Ortlepp, 1937 |
| *Chabertia ovina* | Threlkeld, 1948 |
| *Nematodirus spathiger* | Kates & Turner, 1955 |
| *Ostertagia* spp. | Douvres, 1956 |

The worker must be thoroughly acquainted with the morphological appearance of the moults. In the past the author classified the moult with the stage which follows it (Reinecke, 1963). This is the simplest way of recognizing the various stages but is unacceptable from the biological point of view, where the moult is regarded as part of the previous stage. To overcome this the author has more recently grouped worms in their larval stages as well as moults (Reinecke, 1967; Reinecke & Anderson, 1967). This has not solved the problem, because the moults occupy a very limited period of time in the life cycle and are only recovered in small numbers. The moults should therefore be grouped with the various larval stages, preferably the preceding stage.

By simple proportion the number of each particular stage is estimated by converting the number identified microscopically to the total number of worms present.

11. *Interpreting the results*

Worm burdens in the treated sheep are compared with those in the controls. The dynamic nature of the larval stages and the validity of the Day 0 controls have already been discussed in detail. They can be summarized as follows:—

(1) Sheep with worms one to three days old should be regarded as such and the results expressed in terms of the age of the worms.

(2) Once the worms have reached the fourth or subsequent stage Day 0 controls are valid for comparative purposes with treated sheep killed two or three days later.

<div align="center">DISCUSSION</div>

The methods of carrying out the larval anthelmintic test have been set out in detail. This test has certain advantages.

In the field animals are infested continuously or erratically. Repeated experimental infestations more closely resemble this situation than a single massive dose of infective larvae, after which the animals are treated either at empirical intervals (Banks & Michel, 1960) or when the worms have developed to specific stages (Gibson, 1964).

In all species, with the exception of *O. columbianum*, the third moult is completed within three days. These are regarded as one-day to three-day old worms because they develop rapidly. This group in the test is therefore a modification of the methods of Banks & Michel (1960) and Gibson (1964).

The fourth stage and fourth moult occupy at least six and in some species, e.g. *C. ovina* and *O. columbianum*, twenty or more days. In these groups controls killed on Day 0 indicate the stage of and proportion between the stages on the day of treatment. Moreover, because the rate of development is slower than it is in the previous stage, they are directly comparable with treated sheep killed two or even three days later.

Both these methods, however, avoid the delay of a month between treatment and slaughter used by Gibson (1964). Worm burdens in controls after this long delay are lower than those present on the day of treatment (Reinecke *et al.*, 1962). This possibility is discussed by Gibson but he preferred it to searching for the minute

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worms present in the mass of ingesta when animals are killed soon after treatment. The concentration of worms in small quantities of ingesta has overcome this obstacle (Shone & Philip, 1967; Reinecke, 1967).

Throughout this paper emphasis has been laid on the minimum number of animals necessary for this test, i.e. four treated and four control animals. This preliminary test merely indicates the range of anthelmintic efficacy of the compound and its possible defects. The results should be confirmed on a larger scale, using e.g. fourteen treated and five control animals. At present various statistical methods are being investigated to determine anthelmintic efficacy in at least 80 per cent of a flock. This will decrease the chances of missing anthelmintics which are erratic in their effects against certain worms.

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