

AUSTRALIAN  
STANDARD  
DIAGNOSTIC  
TECHNIQUES  
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STANDING  
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ANIMAL HEALTH  
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STANDARDS

# Anthelmintic Resistance *in Sheep*

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Revised by C. A. Hall (1985) and published by CSIRO for the Australian Agricultural Council (1987) as: Australian Standard Diagnostic Techniques for Animal Diseases, No. 46, *Diagnosis of Anthelmintic Resistance in Sheep and Goats*.

### 1. Introduction

Sheep nematodes have developed resistance to many of the currently available anthelmintics more extensively in Australia than in any other sheep-producing country (Waller, 1986).

The cost of disease caused by worm parasites was estimated at \$A4700 per farm in 1984. Of that cost, 80% was attributed to production losses and the remaining 20% to treatments (Beck *et al.*, 1985). In today's terms, with resistance increasingly widespread and expensive chemicals being used, anthelmintic resistance is the most important problem confronting the sheep industry.

Strategic worm control programs based primarily on using effective anthelmintics are now advocated by all Australian Departments of Agriculture (Anonymous, 1989). Resistance changes with continuous chemical usage: it is not a stable situation. It is central to every worm control program to be able to identify anthelmintics effective for a particular property and to apply information gained about that anthelmintics useful life.

Laboratories adapt procedures to suit their purposes and their techniques undergo continuous modification and improvement. This document provides a starting point to detect anthelmintic resistance and select anthelmintics that are effective for any given property (Table 1).

### 2. Resistance Diagnosis

A clinical response to drenching should occur 7-10 days after treatment. Lack of a clinical response would indicate that anthelmintic failure should be investigated.

The following are steps to be taken to clarify the situation (Fig. 1).

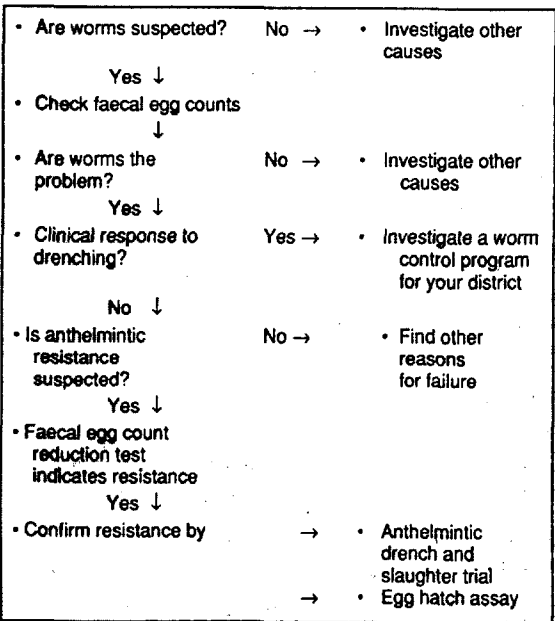


Figure 1. Investigation of a suspected anthelmintic failure.

Table 1. Worm control programs conducted by Australian Departments of Agriculture and Primary Industries

Program	State	Telephone
<i>I. Summer Rainfall Areas</i>		
Wormbuster	Queensland	(07) 362 9447
Wormkill	New South Wales	(067) 73 7248
<i>II. Uniform and Winter Rainfall Areas</i>		
Crack down on worms	Western Australia	(093) 68 3333 (Perth) (098) 42 0500 (Albany)
Drenchplan	New South Wales	(069) 23 0845
Wormcheck	South Australia	(08) 228 7585
Wormplan	Victoria	(053) 37 0755
Wormplan	Tasmania	(003) 41 5218

### 3. Resistance Diagnosed on Faecal Egg Counts

Faecal egg counts are the simplest method of determining worm infestations. If a worm infestation is suspected, faeces for egg counting and culture should be taken. If egg counts indicate that drenching is required, drench and take post-drench faecal samples 10-14 days later to check anthelmintic efficacy.

- At drenching take individual faecal samples from 10 animals in a mob for egg count and culture (see 9. and 10.).
- Drench sheep with an oral anthelmintic (see 13.3.) at the recommended dose rate (*N.B.* Test closantel at one-third the dose rate). Take the following precautions: calibrate the drench gun, drench to the top weight, use clean equipment and ensure that the animal swallows the drench (see 4.).
- Take further faecal samples from these sheep 10-14 days post-drenching for egg count and culture.  
If there is not a 95% or greater reduction in the egg counts after drenching, then anthelmintic efficacy should be further investigated. A pre-drench count of 300-400 eggs per gram of faeces (epg) (average for the group) is sufficient to proceed with the Faecal Egg Count Reduction Test (FECRT, see 5.).
- Larval culture results when interpreted with the egg count data will indicate which species of helminths are exhibiting anthelmintic resistance.

The grazing animal normally carries a mixed parasite infection. The species composition of the population is important. Larval cultures will determine the genera of worms present and their proportion. The choice of anthelmintic will be influenced by the number of resistant genera present.

Egg counts reflect the presence of mature egg-laying worms in the gastro-intestinal tract. If faecal samples for egg count and culture are taken 10-14 days post-drenching, then, after that time, immature worms that have survived the drench will be mature and contributing to the

post-treatment egg count. This time interval will also eliminate the problem of re-infestation. Incoming larvae from the paddock will not be mature by day 14 and their presence will not be reflected in the egg count.

Some drenches, e.g. benzimidazoles, suppress (for up to five days) egg laying by adult worms surviving the drench. By day 10, however, normal egg laying will have resumed.

When evaluating an egg count result, the level of the count, the fecundity and pathogenicity of the helminth and the climatic conditions need to be considered.

Most worm control programs advocate routine faecal egg count monitoring of flocks before and after drenching. If drenches are omitted from a program then regular egg count monitoring becomes essential.

#### 4. Failure to Obtain a Response to Drenching

The lack of response to drenching may be due to several factors including inappropriate drench selection, faulty equipment or drench administration techniques, rather than anthelmintic resistance. Investigate the following options.

##### 4.1. Anthelmintic Choice

Was the correct drench family used? An understanding of anthelmintics and their mode of action is essential for appropriate anthelmintic choice, e.g. a narrow-spectrum drench may not control *Trichostrongylus* or *Ostertagia*.

##### 4.2. Anthelmintic Administration

Did the animals receive the correct volume of drench? Were the animals weighed to calculate dose to body weight? Was the drench administered correctly? Was the drench gun calibrated? Did the animals swallow the drench?

##### 4.3. Parasite Factors

Response to drenching can be masked by the following.

- (a) Larvae unaffected by treatment will continue to develop rapidly and produce signs of disease.
- (b) Rapid re-infection from the pasture. A closantel drench will stop development of incoming *Haemonchus contortus* larvae. Broad spectrum drenches have no effect on incoming larvae (except for sustained release capsules).
- (c) A heavy *H. contortus* burden in weaners will cause severe anaemia and mask the benefits of the drench.

##### 4.4. Concurrent Conditions

Other factors that can hinder recovery are:

- (a) ill thrift due to poor nutrition; and
- (b) bacterial or protozoan diseases, e.g. coccidiosis, salmonellosis, eperythrozoonosis.

*Eperythrozoon ovis* is a blood protozoan that causes anaemia in weaner sheep. Tolerance to mustering is also reduced. Most outbreaks are observed during late winter and spring in high rainfall areas. Mosquitoes may transmit the parasite. Affected sheep should be handled carefully, given nutritious feed and a good water supply, sheltered from the elements and disturbed as little as possible.

#### 5. Resistance Diagnosed on a Faecal Egg Count Reduction Test

The FECRT is the most practical method of determining resistance to anthelmintics. It allows any number of drenches to be tested at the same time and produces valuable information for planning drench rotation systems on individual properties.

This test should be performed when adopting a strategic worm control program. Alternatively, if the adopted worm control program appears not to be performing, then the FECRT is recommended to reassess anthelmintic resistance on the property in question.

The FECRT answers the question — Will this drench kill all the worms in my sheep? There are two aspects to this procedure: the field trial (see 5.1.) and the laboratory procedures (see 5.2.).

##### 5.1. Field Trials

###### 5.1.1. Planning

###### 5.1.1.1. Age of test sheep

Sheep should be three to six months old. Egg counts are too low in younger animals. In older animals, an increasingly skewed egg count distribution results from different rates of acquisition of immunity. This leads to potentially large differences between group mean counts, invalidating the comparison of the control with the test groups.

###### 5.1.1.2. Number of test sheep

At least 15 sheep per drench group with faecal sampling of at least 10 need to be set aside for up to two weeks.

###### 5.1.1.3. Drenching history of test sheep

Sheep should not have been drenched with a broad spectrum drench in the last four weeks and preferably the last 10 weeks or with a closantel drench in the last 10 weeks. Undrenched sheep are preferable.

###### 5.1.1.4. Worm burden of test sheep

A preliminary egg count of at least 200 epg (group average) is a prerequisite for a FECRT to be performed. Some programs require 500 epg.

###### 5.1.1.5. Drenches to be tested

(See 13.3.) BZ/levamisole/BZ:levamisole combination/ivermectin/naphthalophos/ closantel and/or capsule. For each property, all appropriate drenches should be tested. In subsequent tests

a product to which extreme resistance was previously detected, may be omitted on the basis that reversion to susceptibility would not have occurred.

#### 5.1.1.6. Time of the year

Tests performed soon after a summer drought may overestimate the degree of resistance on that property. The population of worms in the host are the resistant survivors of the last drench. No larval pickup from the paddock, due to the drought, would have occurred to reveal the true situation for that property.

#### 5.1.2. Equipment

- (a) Scales to weigh the sheep (bathroom scales are adequate).
- (b) Colour marking paint (e.g. SIROMARK or easily scourable dry raddle) to indicate drench groups on the sheep.
- (c) Faecal collection bottles or plastic bags.
- (d) A permanent marking pen for labelling collection containers. Drench gun(s) and back-pack.
- (e) Associated paper work, i.e. Laboratory Advice Sheets.

#### 5.1.3. Operation

##### 5.1.3.1. On day 1 of the test

5.1.3.1.1. Select suitable sheep. Draft off enough sheep to allow 15 per drench group — six drench groups plus one control group ( $6 \times 15$ ) + 15 = 105 lambs. They should be this year's lambs, of even size and preferably not previously drenched.

5.1.3.1.2. Determine weight for drench dose calculations. First exclude atypically heavy sheep. Weigh five of the heaviest looking sheep in the draft. Use the heaviest weight for all dose calculations.

5.1.3.1.3. Randomise sheep into drench groups. A form of systematic randomisation is used. Sheep in the race are allocated into groups on the basis of No. 1 to the first group, No. 2 to the second group and so on. The common practice of allocating the first 10 sheep into the first group, the second 10 into the second group and so on does not constitute proper randomisation. Colour mark the sheep sequentially down the race using head, neck, back and rump marks until all sheep are accounted for. There should be 15 sheep per group.

5.1.3.1.4. Drench. Dose sheep at the following rates (Table 2). (N.B. Doses vary depending on the product: check the label.)

At one-third dose rate, the residual activity of closantel is eliminated. The efficacy of the drench against the host worm burden can then be assessed.

##### 5.1.3.1.3. Hints

- (a) Use the clear drenches before the white drenches.
- (b) Rinse and clean drench guns and packs with water between each test drench.

**Table 2.** Anthelmintic dose rates for sheep in the Faecal Egg Count Reduction Test

Product	Rate
Levamisole	1 mL per 10 kg
Closantel <sup>1</sup>	1 mL per 15 kg
Combination	1 mL per 10 kg
Benzimidazole	1 mL per 12 kg
Ivomectin	2.5 mL per 10 kg

<sup>1</sup> Closantel is used at one-third its normal dose rate.

- (c) Calibrate drench guns before using each test drench, i.e., squirt 10 doses into a measuring cylinder.

##### 5.1.3.2. On day 2 of the test

The second day of the test is 10–14 days after the 'test' drenching.

5.1.3.2.1. Collect faecal samples. Collect faecal samples individually into collection containers from all sheep. Containers must be marked according to the groups. Mark owner's name on the transport box/plastic bag and fill in Laboratory Advice Sheet.

5.1.3.2.2. Submit faecal samples to the laboratory. Faecal samples should be submitted immediately to the laboratory together with the completed Specimen Advice Sheet.

Where transport will take longer than overnight, despatch samples in an esky containing a freezer brick wrapped in several layers of newspaper. N.B. Storage of samples below 4°C will prevent *Haemonchus contortus* eggs from hatching.

#### 5.2. Laboratory Procedures

##### 5.2.1. Processing of Samples

- (a) Use one of the bulk egg counting techniques (see 9.2.) to process faecal samples into drench groups. Some laboratories prefer to use individual counts (see 9.1.).
- (b) Prepare one bulk larval culture per drench group (see 10.).
- (c) Read larval cultures seven to eight days later. See Key I (10.2.) for larval differentiation.

##### 5.2.2. Calculation of Results

- (a) The recommended procedure for the conduct of a FECRT is published in Anonymous (1989). The worked example is adapted from that information (see 5.2.2.1). Two computer programs have been developed by CSIRO (Martin and Wursthorn, 1990) to assist with the statistical calculations and interpretation of FECRTs. The first, RESO.WKI is a worksheet for use with Lotus 1-2-3. The second, RESO.EXE is an executable program for use with the computer DOS. Both programs are for IBM or compatible machines.

**Table 3.** Faecal egg count reduction test — worked example (post treatment counts, 10 days)

Group No. (i):	Faecal egg count in eggs per gram		
	Control	Anthelmintic A	Anthelmintic B
	1	2	3
Sheep No. (j):			
1	525	15	180
2	450	0	135
3	270	0	510
4	540	30	180
5	90	0	105
6	765	0	225
7	120	0	390
8	945	0	210
9	465	45	15
10	255	0	150
No. in group <sup>1</sup>	10	10	10

<sup>1</sup> Total number of sheep in the group =  $n_i$ ; ( $N = \sum n_i$ )

Arithmetic mean count  $\bar{X}_i = \sum_j X_{ij} / n_i$

Variance of counts  $s_i^2 = [\sum_j X_{ij}^2 - (\sum_j X_{ij})^2 / n_i] / (n_i - 1)$

Per cent reduction  $R = 100(1 - \bar{X}_i / \bar{X}_c)$

Variance of reduction (on log scale)

$V = [(s_i^2 / (n_i \bar{X}_i^2)) + (s_c^2 / (n_c \bar{X}_c^2))]$

Approximate 95% confidence interval for R

$100[1 - (\bar{X}_i / \bar{X}_c) \exp(\pm 2.1\sqrt{V})]$

Upper confidence limit  $100[1 - (\bar{X}_i / \bar{X}_c) \exp(-2.1\sqrt{V})]$

Lower confidence limit  $100[1 - (\bar{X}_i / \bar{X}_c) \exp(+2.1\sqrt{V})]$

where  $s_i^2$  denotes the variance on the arithmetic scale

calculated as above or  $s_i^2 = \sum_j (X_{ij} - \bar{X}_i)^2 / (n_i - 1)$

- (b) Resistance is said to exist if the reduction in the arithmetic mean from the drench group is less than 95% when compared with those from untreated sheep, and in which the lower 95% confidence limit is at less than the 90% reduction level.

#### 5.2.2.1. Worked example

Post treatment counts (10–14 days) (Table 3).

### 6. Resistance Diagnosed on an Anthelmintic Drench and Slaughter Trial

The *in vivo* drench and slaughter trials, because of their magnitude and cost, are used as investigational or research tools only (Powers *et al.*, 1982).

Strains of 'suspect resistant' larvae are produced in culture. Sufficient numbers of these larvae can also be produced by passage through a producer lamb. The response of these larvae to the test anthelmintic is compared with the responses of known susceptible and known resistant nematode larvae for that particular anthelmintic. Anthelmintics are usually tested at three dose rates.

Results are compared statistically to determine if changes in the angle and position of the response lines are significant.

#### 6.1. Worm-free Host Lambs

- (a) Host lambs should be less than seven months old, reared on concrete and fed a prepared ration.

Alternatively, purchased lambs of suitable age and carrying low worm burdens can be drenched with ivermectin at twice the therapeutic dose rate on two occasions, one week apart. Egg counts by the McMaster method (see 9.1.) must be zero prior to the commencement of the trial. Leave at least seven days after the second dose of ivermectin before infecting with the suspect larvae.

- (b) A minimum of four worm-free lambs (one control and one per dose rate of the anthelmintic) are required for the procedure; however, six animals per dose group are preferable. One 'producer' lamb for 'suspect resistant' larvae production may also be required.

#### 6.2. Production of Infective Larvae

- (a) Culture faeces from animals carrying the 'suspect resistant' worms. The amount cultured will depend on the egg count. The numbers of infective larvae required for the trial are about:

2500 — 5000 *Haemonchus* spp.

2000 — 3000 *Ostertagia* spp. or

3000 — 6000 *Trichostrongylus* spp.

Alternatively, if insufficient larvae are available, infect the 'producer' lamb with all of the available larvae. Collect faeces from this lamb 28 days post infection if egg counts are sufficiently high. Culture the collected faeces to produce the required number of 'suspect resistant' larvae.

#### 6.3. Infection of Worm-free Lambs

- (a) Infect the lambs by intraruminal injection with the required number of infective larvae suspended in water.
- (b) Determine egg counts of infected lambs between days 23 and 27.
- (c) Weigh each animal.
- (d) Allocate lambs into groups by stratified randomisation according to body weight and faecal strongyle egg counts.

#### 6.4. Testing the Drench

- (a) On days 24–28 after infection, treat all animals with the appropriate 'test' drench (Table 4). A second anthelmintic, with another mode of action should be tested on another set of sheep.
- (b) Autopsy all animals 7–10 days after treatment. Collect the appropriate portion of the gastro-intestinal tract. Process each section separately (see 11.).

**Table 4.** Dose rates for drenches to be tested

Group No.	Test	Rate (x recommended dose rate)
1	Anthelmintic	0.5
2	Anthelmintic	1.0
3	Anthelmintic	1.5
4	No treatment	Control

### 6.5. Calculation of Results

- Calculate the per cent efficiency. For each anthelmintic compare the average number of worms per dose rate with those of the control group.
- Produce dose response lines (Clark and Turton, 1973).
- Compare these lines of dose response with those produced for the same anthelmintic against susceptible strains of the same nematode species.
- Resistance is diagnosed if the change in the angle of the response and the position of the response is significantly different from the susceptible strain and similar to the resistant strain.

## 7. Resistance Diagnosed on Egg Hatch Assay

Benzimidazole (BZ) anthelmintics prevent nematode egg embryonation and hatching. This characteristic can be used to detect BZ resistance (Le Jambre, 1976). Eggs are collected immediately from fresh faeces and incubated in serial concentrations of the BZ anthelmintic. A pure strain of nematodes is desirable.

### 7.1. Reagents

- Dissolve 8 mg of pure TBZ (Thiabendazole) in 5 mL of dimethyl sulfoxide (DMSO) (see 13.3.). Make to 1 L with 0.1% sodium chloride (0.017 mol/L) (stock solution).
- Make serial dilutions of TBZ, ranging in concentration from 3.0 to 0.007 µg/mL using a standard diluent of 5 µL/mL DMSO in 0.1% sodium chloride.
- Pipette 1 mL of TBZ serial dilutions into each well of a 25-well petri dish (Sterilin No. 234095).

### 7.2. Egg Recovery

- Collect 50 g of faeces directly from the animal(s) infected with the strain to be tested. A pure infection is required.
- Process faeces immediately. Do not chill.
- Mix faeces with water to a slurry.
- Pour through two thicknesses of surgical gauze or muslin into a 3 L side-arm flask.
- Wash the slurry again with 1 L of saturated sodium chloride solution.
- Discard gauze and debris. Evacuate the sieved sample with a tap aspirator to remove bubbles.
- Sieve evacuated sample into a plastic photographic tray. Gently place a perspex sheet, cut to fit the tray, on the surface of the sample.
- Store at 4°C for 15–20 min. Lift up perspex sheet and wash off adhered eggs into a 250 mL cylinder.

- Add chilled water up to the mark and stand at 4°C for 90 min.
- Decant the supernatant to a volume of 50 mL. Further decant in a 50 mL cylinder or centrifuge at 2000 rpm for five minutes to concentrate eggs.
- Adjust the volume of the egg suspension to 200 eggs/0.1 mL. Add the 0.1 mL to the 1 mL TBZ solutions and the control well.
- Incubate at 22°C for 48 hours. Terminate with a drop of dilute iodine solution.
- Count eggs and first stage larvae in each well under x40 magnification. Count a duplicate.
- Calculate per cent hatch.

### 7.3. Statistical Analysis

For each TBZ concentration, the proportion of eggs killed by the TBZ alone (P) is calculated from Abbott's formula which adjusts the total proportion killed (P') in relation to the number dying in the TBZ free control sample (C) by:

$$P = (P' - C)/(1 - C) \text{ (Finney, 1971).}$$

The percentage of unhatched eggs at each concentration of TBZ is converted to probits and plotted against the logarithm of the TBZ concentration. A log concentration-probit (lc-p) line can be fitted using a computer program written following the methods published by Davies (1971). The lethal concentration to 50% of eggs (LC<sub>50</sub>) and the resistance ratio [RR = exp(log LC<sub>50</sub> test strain - log LC<sub>50</sub> susceptible strain)] gives quantitative measures of resistance. The 95% confidence intervals for a resistance ratio can be calculated from the formula:

$$\exp[\log(RR) \pm 2 * (S_1^2 + S_2^2)]$$

where S<sup>2</sup> is the variance for each strain from the probit analysis. (PROBIT is an IBM PC computer program for probit analysis. Copies of PROBIT are available from: Leo Wursthorn, CSIRO, Animal Health Research Laboratories, Private Bag No. 1, Parkville, Vic. 3052, Australia).

## 8. Broad Spectrum Anthelmintic Resistance Diagnosed by *in vitro* Larval Development Assay

The Larval Development Assay (LDA) is an *in vitro* technique for detection of broad spectrum anthelmintic resistance in nematodes. Each row of 12 wells, of a 96-well microtitre plate, contains a 1000-fold concentration range of a specific anthelmintic in an agar matrix. At present, the anthelmintics used are specifically for detection of benzimidazole, levamisole/morantel and ivermectin resistances.

Nematode eggs are isolated from a faecal sample, applied to the wells and allowed to develop to infective L<sub>3</sub> larvae over six to seven days. Eggs in wells will hatch and develop through L<sub>1</sub> and L<sub>2</sub> stages depending on concentration of the

anthelmintics. Thus, isolates resistant to an anthelmintic will develop in wells containing higher concentrations than susceptible isolates (Lacey *et al.*, 1991).

The LDA offers the following advantages over existing techniques:

- simultaneous evaluation of all broad spectrum drenches in a single assay;
- single farm visit with minimal on-farm experimentation; and
- eliminates between-animal variation as a source of poor data quality to give improved precision of resistance status.

The LDA is currently under field evaluation for commercial development by Horizon Agriculture and CSIRO, Division of Animal Health.

## 8.1. Field Collection

- Randomly select at least 10 animals from the flock to be tested and collect no less than 100 g of faeces as a pool sample.
- Take a representative egg count on the bulked sample. Submit samples with an  $\text{epg} > 100$  for LDA.
- Gently press the faeces to exclude air and seal tightly in a plastic container. Do not crush pellets into a single mass.
- Hold and transport sample at room temperature. The time between collection and assay should be less than seven days.

## 8.2. Laboratory Procedure

- Eggs are isolated by modified sucrose flotation technique.
- Eggs are applied to pre-prepared plate and incubated for seven days at 22°C by which time control (no drug) wells have developed to infective L<sub>3</sub> larvae.
- Larvae are killed with iodine and development assessed by two methods:
  - Qualitative* (by eye). Transition between L<sub>3</sub> (uninhibited) and L<sub>1-2</sub> (inhibited) wells can be assessed by eye to identify well number at which about 50% inhibition of development occurs.
  - Quantitative* (counting). Proportions of L<sub>1-2</sub> and L<sub>3</sub> larvae (and eggs) can be counted and data computer-fitted by logit-log concentration model to derive LD<sub>50</sub>.

## 8.3. Calculation of Results and Interpretation

- Approximate LD<sub>50</sub> values for a field isolate can be obtained by conversion from well number using the concentration factor  $b$  where  $b$  is the concentration in well No. 1, thus:

$$\text{LD}_{50} \text{ (in } \mu\text{mol/L)} = a \times b$$

where  $a$  is well number in which inhibited L<sub>1-2</sub> larvae and uninhibited L<sub>3</sub> are in roughly equal abundance. *N.B.* Qualitative LD<sub>50</sub>s normally agree within two-fold of the computed quantitative value.

- Resistance factors (RFs) can be calculated by:

$$\text{RF} = 2^{(a-c)}$$

where  $a$  and  $c$  are the well numbers in which approximate LD<sub>50</sub> values of field and susceptible isolates are noted. Alternatively, if a susceptible isolate is not run with the field isolate, RF can be calculated as:

$$\text{RF} = [(a \times b) / \text{SLD}_{50}]$$

where SLD<sub>50</sub> is the data base susceptible value and  $b$  is concentration factor.

- The RF for a field isolate is used with the accompanying data base to predict the faecal egg reduction achievable for the field isolate. The data base has been constructed using 40 sets of field FECRT and LDA data. For more precise property monitoring, the LDA RF values can be used initially in conjunction with a FECRT in year 1 to establish correlation on the property, then subsequently monitored using LDA.

## 9. Counting Techniques for Strongyle Eggs

Strongyle eggs are floated in a known volume of faecal suspension and then counted microscopically on a Whitlock Universal or a McMaster slide. Direct extrapolation of the number of worms to be found in the gut from the egg of faeces calculation is only approximate. Egg production is influenced by many things, e.g. immunity of the host, species of worm, maturity of worms, season of the year and stage of pregnancy and lactation.

Egg counts can give valuable information on existing worm burdens and larval paddock populations if samples are taken just prior to drenching and on anthelmintic efficacy if faecal samples are taken 10–14 days post drenching.

### 9.1. The Modified McMaster Method

This method is based on the McMaster Method (Whitlock, 1948) and uses a Whitlock Universal (4 x 0.5 mL) or a McMaster (3 x 0.3 mL) slide and is the standard procedure adopted for egg counting in individual animals (McMaster and Whitlock Universal egg counting chambers are available from: J. A. Whitlock and Co., PO Box 51, Eastwood, NSW 2122, Australia). A variation of this method uses a bulking technique prior to mixing for the batch processing of large numbers of samples.

For liquid or soft faeces only, discard the top layer of the faecal sample (0.5 g). Mix the remainder. No correction for faecal consistency is necessary for FECRT samples. Correct randomisation of animals will allow for variability in faecal consistency.



- (a) Weigh 2 g of faeces into a 60 mL mixing jar. To soften faecal pellets, add 2.5 mL of tap water or 2.5 mL of 0.1% aqueous methylene blue to each jar, roughly break up the pellets with a pair of forceps and soak, covered, for one hour or refrigerate overnight.
- (b) Add 47.5 mL of flotation fluid usually saturated sodium chloride solution (specific gravity 1.20) to each jar. Some laboratories use saturated magnesium sulfate (see 13.1.).
- (c) Mix with a mechanical mixer or a hand held kitchen mixer, until the faeces are broken up and well dispersed.
- (d) Mix and stir the suspension violently with a sieve ended pipette (wire gauge of 12 meshes per cm) until a homogenous solution is obtained. One or two drops of amyl alcohol [2-methyl-2-butanol,  $C_2H_5C(CH_3)_2OH$ ] will disperse any bubbles. *N.B.* Amyl alcohol can be an irritant.
- (e) Pipette an aliquot from the centre of the suspension into two chambers of the counting slide. Note well the following points.
  - (i) Drain pipette before collecting the sample.
  - (ii) Lightly bounce the pipette while filling to prevent blockage of sieve by fibre.
  - (iii) Tilt the mixture to allow maximum filling of pipette.
  - (iv) Dab the end of pipette with tissue after filling to remove any bubbles from the surface of the mixture.
  - (v) Ensure that the pipette is held horizontally and fill the chamber in one action without producing bubbles. Allow the eggs from 1 to 10 min to float up under the glass before counting.
- (f) Count the eggs in the chambers using x40 magnification. Count all eggs within the double line boundaries.
- (g) Calculate the epg of faeces by multiplying the number of the eggs counted by the total volume (50 mL) divided by the volume counted times the weight of the faeces (Table 5). If other counting slides are used, make appropriate adjustments for volume when calculating multiplication factors. A minimum of two chambers should be counted unless egg counts are very high (>2000 epg). Eggs of *Nematodirus*, *Trichuris*, *Strongyloides* and tapeworms are easily identified (Soulsby, 1965). *Nematodirus* eggs

should be counted separately from strongyle eggs. Coccidial oocysts can also be counted.

- (h) Usually 10 animals in a group are sampled and the faeces are processed individually resulting in 10 egg counts.

### 9.2. Composite Bulking Technique

A variation of the modified McMaster method (Baldock *et al.*, 1990) uses a bulking technique prior to mixing for the batch processing of large numbers of samples. This method will give a value approximating the arithmetic mean of individuals within a group.

From any group of 10 samples, two counts are produced.

- (a) Weigh 0.5 g from each of five samples into the jar to make a 2.5 g sample. Repeat the procedure for the next five samples from the group of 10 samples.
- (b) Process each of the two composite samples as for the McMaster method above.
- (c) The multiplication factor is x40 to express the result as epg. If fewer than 10 samples are to be processed the table below should be consulted for the multiplication factors (Table 6).

*N.B.* There are a number of bulking techniques being used in Australian diagnostic laboratories. Many laboratories regard 0.5 g of faeces/animal as too small a sample and prefer to use 2 g to eliminate the potential for 'error' due to non-random mixing of eggs in faeces.

### 9.3. Centrifugation Method

This modification will detect eggs in animals infected with species of low fecundity such as *Ostertagia*. It also allows storage of partly processed samples (up to the salt stage). Very few air bubbles are produced by this method.

- (a) Weigh 1.5 g of faeces from each sample into a 60 mL container.
- (b) Add 28.5 mL of clean tap water from a dispenser to each sample. Allow to soak for a few minutes to one hour to soften the faeces.
- (c) Homogenise each sample using a laboratory stirrer or glass beads.
- (d) Pass the homogenised sample through a small household tea strainer mounted over a suitably sized bowl to collect the liquid.

Table 5. Volume of Whitlock Universal Chamber = 0.50 mL

No eggs counted :	In 1 chamber	In 2 chambers
Total volume	50 mL	50 mL
Volume counted (chamber) <sup>1</sup>	0.5 mL	1.0 mL
Weight faeces	2 g	2 g
Multiplication factor	x50	x25

<sup>1</sup> Whitlock Universal chamber.

Table 6. Multiplication factor based on pooling 0.5 g aliquots and reading total eggs in one chamber of volume 0.5 mL

No. 0.5 g aliquots pooled	Multiplication factor if one chamber
3	x66
4	x50
5	x40
6	x34

- (e) Swirl to mix the liquid, then pour immediately into a 15 mL numbered centrifuge tube. Excess liquid can flow over the tube once it is full.
- (f) Centrifuge for two minutes at 2000 rpm to produce a plug of debris containing the eggs. Pour off the supernatant carefully or remove it using a water-driven suction pipette.
- (g) Add saturated sodium chloride solution up to the 10 mL mark. Resuspend the plug by repeated inversions of the tube. Fill the tube up to the 15 mL mark with more saturated sodium chloride solution and mix thoroughly using inversion or a pasteur pipette.
- (h) After mixing the faecal suspension, fill the chamber of the Universal Whitlock Slide (0.5 mL in each chamber). Count all the eggs within the first two lines of the chamber. The multiplication factor is 100. If few eggs are present, count chambers one and two to give a sensitivity of 20 epg.
- (i) Wash the stirrer, the mixing container, the sieve and the collecting bowl between samples.

## 10. Faecal Culture and Identification of Nematode Larvae

The value of a faecal egg count is increased if the species of worms present can be identified. While most strongyle eggs are similar in shape and size and not readily identified, infective larvae are morphologically distinct and readily differentiated.

Faecal cultures provide an environment suitable for the hatching of helminth eggs and development to the infective larval stage.

### 10.1. Setting Up the Culture

- (a) For an individual culture, transfer about 20 g of faecal material to a culture bottle. A 250 mL glass or a disposable polystyrene toxicology jar is adequate for small cultures. For a bulk culture, add about equal amounts of faecal material (3–5 g) from each individual sample to the culture bottle to make a 30 g sample.
- (b) Add 20 mL of water. Use more water for larger amounts of faeces. Mix with a hand-held kitchen mixer. Add 5 g of vermiculite No. 3 (Medium Grade) (Vermiculite available from: Neuchatel Trading Co., 6 Mackie Way, Brendale, Qld 4500). Mix lightly with forceps. Do not pack the mixture. Alternatively, mix faecal material in a clean mortar. Add vermiculite and some water. Mix with a gloved (plastic) hand or pestle to give a crumbly mixture. Transfer to a labelled culture bottle. Rinse down the sides of the culture bottle with a small amount of water. Do not pack the mixture. *N.B.* The moisture content of a culture is important.

A high moisture content may lead to massive fungal growth at the expense of larval survival. If faeces are dry, use less vermiculite.

- (c) Place the lid (minus wad if applicable) on the bottle, turn lightly but do not seal.
- (d) Incubate at 27°C for seven days.
- (e) After incubation, expose the culture to light for one hour. Then fill the culture bottle with warm water (30°C) and invert in a glass Petri dish. Fill the moat thus formed with water. Stand for three to eight hours until larvae collect in the moat. Pipette off liquid plus larvae into a centrifuge tube. *N.B.* Some laboratories use a Baermann funnel to separate larvae. Larvae can now be stored for several weeks at 4–10°C if necessary.

### 10.2. Reading the Culture

- (a) Allow larvae to sediment in the centrifuge tube. Discard some supernatant with a vacuum pipette. The amount discarded will depend on the density of the larvae.
- (b) Mix larvae, pipette a drop of the suspension onto a microscope slide. Add one drop of Parasitological iodine (see 13.1.) to kill and stain the larvae. Cover with a coverslip (40 x 22 mm) and examine under a microscope at x100 magnification. Heat fixing will straighten the larvae allowing measurement.
- (c) Count 100–200 larvae, differentiate into species (see Key I, Table 7 and Figs 2a and 2b). Express results as a percentage for each species.
- (d) Larvae of *Ostertagia circumcincta* and *Trichostrongylus* spp., particularly *T. vitrinus* can be identified after exsheathing in sodium hypochlorite (NaOCl) solution (photographic concentration). Larvae of *T. vitrinus* may measure up to 796 µm.

**Key I.** Identification of the 3rd stage larvae of some common gastro-intestinal nematodes of sheep

1.	Oesophagus rhabditiform	Free living nematode
	Oesophagus not rhabditiform	2
2.	Without sheath, oesophagus nearly half the length of the body	<i>Strongyloides</i>
	With sheath, oesophagus less than 1/4 the length of the body	3
3.	Tail of sheath short or of medium length	4
	Tail of sheath very long	7
4.	Two refractile bodies or a bright transverse band visible between buccal cavity and oesophagus	<i>Cooperia</i>
	Refractile bodies or band absent	5
5.	Slender larva, tail of sheath of medium length tapering to a point and often kinked	<i>Haemonchus</i>
	Tail of sheath very short, conical	6
6.	Larva of medium size or large with distinct rounded tail	<i>Ostertagia</i>
	Small larva, tail bearing one or two tuberosities or indistinctly rounded	<i>Trichostrongylus</i>
7.	Very large larva, 8 gut cells, tail notched, bilobed or trilobed	<i>Nematodirus</i>
	Larva of medium size, 16–24 pentagonal gut cells, lumen of gut wavy	<i>Oesophagostomum</i>
	Larva of medium size, 24–32 square gut cells, lumen of gut straight	<i>Chabertia</i>
	Very small larva with 16 gut cells	<i>Bunostomum</i>

**Table 7.** Measurement of infective nematode larvae of sheep (adapted from Dikmans and Andrews, 1933)<sup>1</sup>

Length, end of larva to end of sheath (µm)	Species, with range of total length (µm)	Key to Figure 2	Other differentiating features
No sheath	<i>Strongyloides</i> 570–700	A	Slender body with long oesophagus, 1/3 to 1/2 total length of larva.
Long 85–115	<i>Bunostomum</i> 510–670	B	Wide body with sudden tapering to long thin tail. 'Band' constriction on oesophagus.
Short 20–40	<i>Trichostrongylus</i> 620–720	C	Short straight larva, conical tail sheath. <i>T. colubriformis</i> has tubercles <sup>2</sup> on tail of larva. <i>T. axei</i> , no tubercles on tail.
	<i>Ostertagia</i> 700–910	D	Longer, conical, 'finger-like' sheath.
Medium 40–80	<i>Cooperia curticei</i> 710–850	E	Oval bodies at anterior end of larva. Tail of larva rounded, but not obvious.
	<i>Haemonchus</i> 650–750	F	Tail sheath is usually 'kinked'. Pointed tail of larva.
	<i>Cooperia oncophora</i> 800–920	G	Oval bodies anterior end of larva. Tail of larva rounded, but not obvious.
Long 110–160	<i>Chabertia</i> 710–790	H	Stout body with 24–32 rectangular intestinal cells.
	<i>Oesophagostomum</i> 770–920	I	Usually longer than <i>Chabertia</i> . Has 16–24 triangular intestinal cells.
Extremely long 250–290	<i>Nematodirus</i> 922–1180	J	Very big larvae with very long tail. Tail of larva is forked.

<sup>1</sup> Strain variations exist around this mean. <sup>2</sup> After exsheathing in sodium hypochlorite (photographic concentration).

## 11. Total Worm Count — Gastro-intestinal Nematodes

Faecal egg and larval counts do not always give reliable information about worm burdens except in very young animals. Examination of the intestinal tract will provide information about the size of the burden and the stages present. All techniques are based on a sieving and aliquot technique, and the one described is an example. Refer to Powers *et al.* (1982) for further total worm counting procedures.

### 11.1. Equipment

Plastic trays 50 x 35 cm

Wide-mouthed screw-top plastic jars of about 500 mL capacity

Wide-mouthed 2 and 4 L mixing jars

Sieves (Endecotts Ltd test sieves are available from: Selby Scientific Ltd, Melbourne, Vic., Australia.)

Scoops

Pump for aeration

Formalin

Parasitological iodine (see 13.1.)

Saturated sodium thiosulfate (see 13.1.)

### 11.2. Autopsy

- Deprive animals of food for 24 hours before autopsy.
- Isolate the gastro-intestinal tract into its component parts as soon as possible after death. Strip off adipose tissue and mesenteric attachments.

- Locate and tie off each organ separately with string before excising from the tract.
- If organs cannot be processed immediately, refrigerate for up to 12 hours. Place each organ in a separate plastic bag and label with the relevant information, e.g. eartag No., date of slaughter, etc. Alternatively, embalming fluid (see 13.1.) will preserve the contents but leave the gastrointestinal tract in a pliable condition and able to be 'run'.

### 11.3. Worm Recovery

Each organ is processed separately.

#### 11.3.1. Abomasum

- Open the organ along its greater curvature and spill the contents into an 80 mesh sieve. If larval parasites are present, a 400 mesh sieve should be placed under the 80 mesh screen. Wash out as much material as possible into the sieve with a gentle jet of water.
- Spread the abomasum on a flat tray, mucosa up and scrape off attached worms with the gloved hand and a jet of water. Remove worms from both sides of the abomasal folds. Add washings to the sieve.
- Back wash into a sealed plastic container. Add buffered formalin (CH<sub>2</sub>O) to give a final concentration of 5% formalin. Some laboratories prefer 10% formalin.
- Refrigerate the abomasal wall for digestion techniques (see 11.4.) if infection with histotropic stages is suspected.

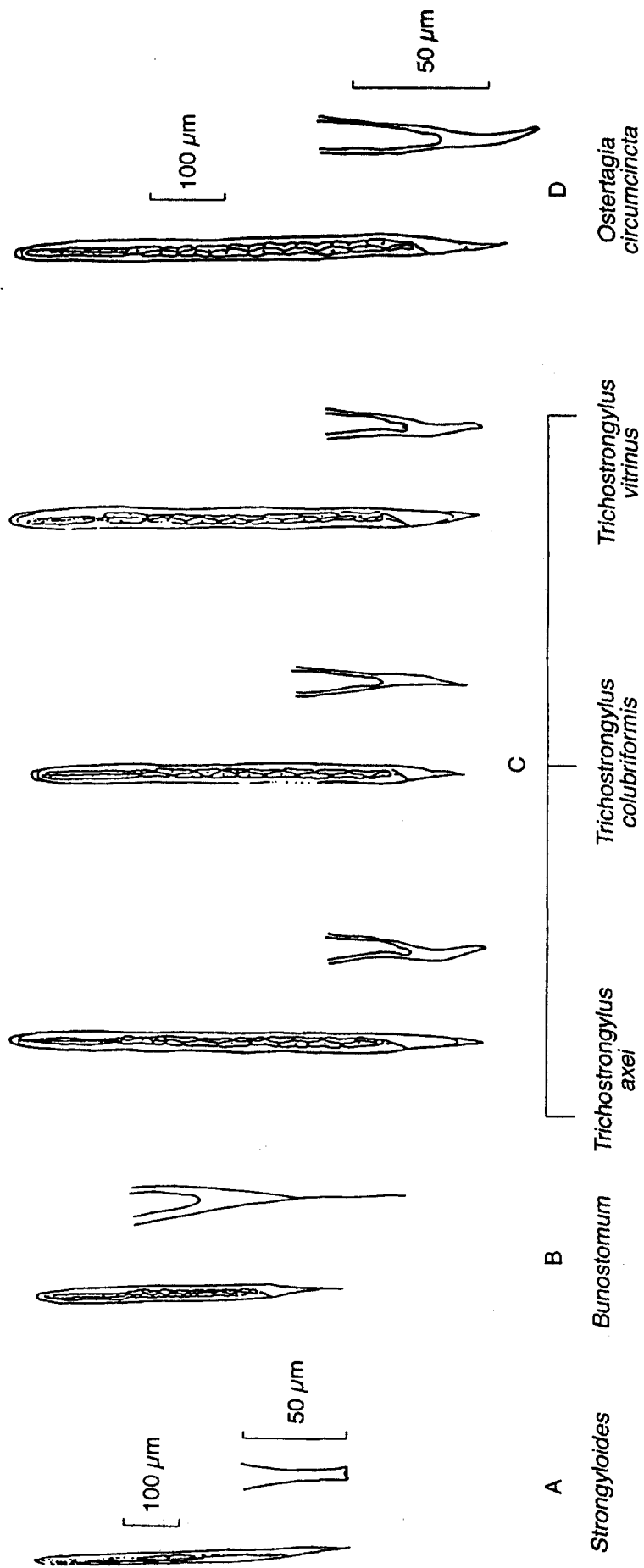


Figure 2 (a). Infective larvae of parasitic nematodes of sheep (Ministry of Agriculture, Fisheries and Food, 1977).

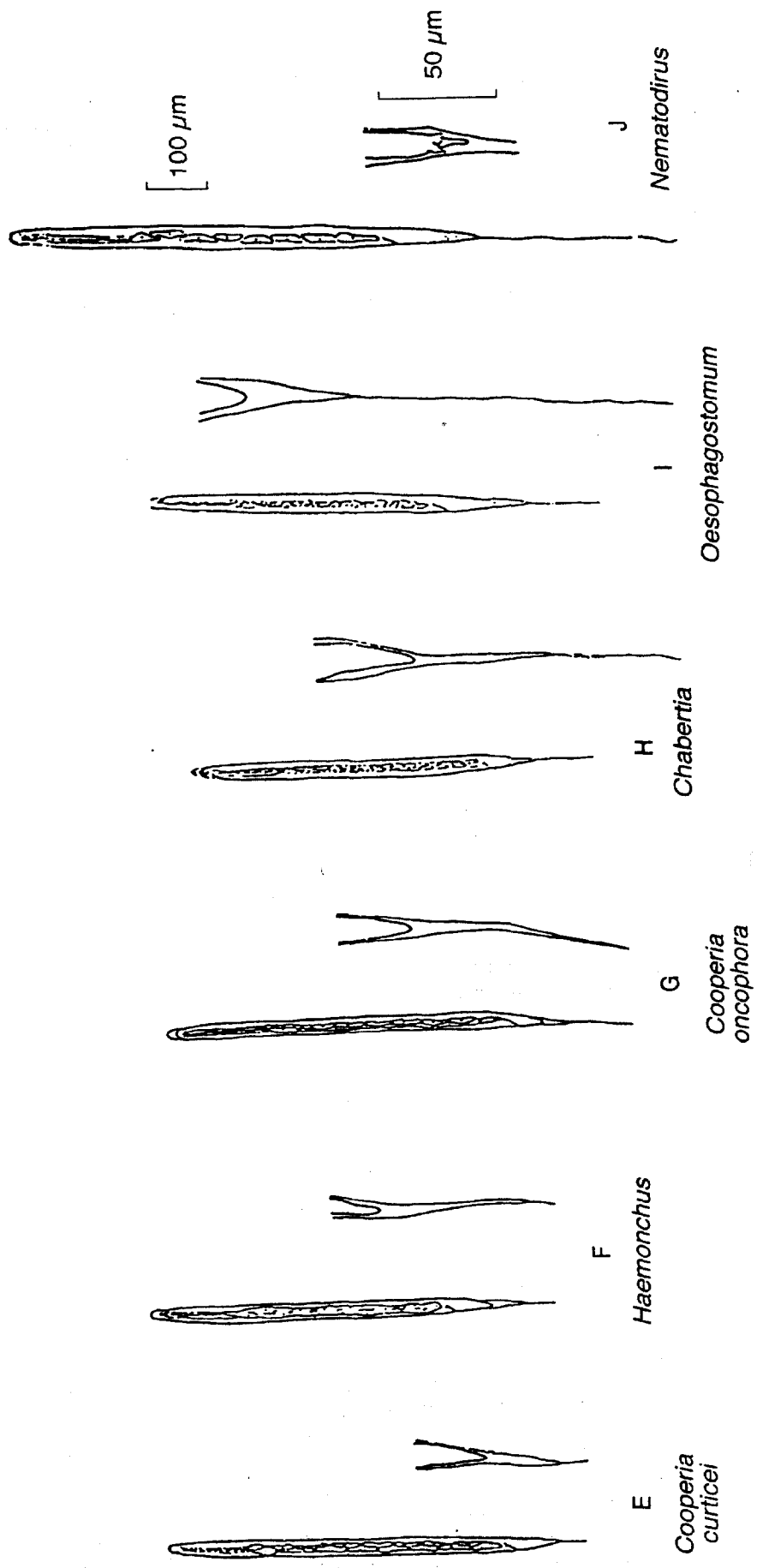


Figure 2 (b). Infective larvae of parasitic nematodes of sheep (Ministry of Agriculture, Fisheries and Food, 1977).

### 11.3.2. Small Intestine

- (a) Strip the intestine from the mesentery and remove all excess fat. Most worms are found in the upper half of the small intestine.
- (b) Cut open the small intestine, squeeze the contents off between thumb and forefinger and collect by washing onto a 100 mesh sieve. Alternatively, a 'gut runner' (see 13.2.) will open and scrape the mucosa in one operation. The gut can also be run unopened using the fingers to squeeze the contents out onto the sieve. Wash through the gut twice with water.
- (c) Collect and preserve the contents as for the abomasum. Refrigerate small intestinal wall for digestion if necessary.

### 11.3.3. Large Intestine

- (a) Open the rectum and remove enough faeces for a faecal egg count and culture.
- (b) Open the organ onto a tray, wash with tap water and add contents to a 60 mesh sieve. The species present are large and readily seen.

### 11.4. Digestion Technique for the Recovery of Immature Nematodes

- (a) Process the abomasal tissue and sections of the small intestine separately. The mucosa is normally scraped off the abomasum with a knife or glass slide.
- (b) Transfer mucosal scrapings or small intestine sections to a large wide-mouthed jar. Add 1 L of digest liquid. Loosely place lid on jar.
- (c) Incubate at 40°C for two to four hours. Stir frequently. Estimate the end-point visually.
- (d) Remove tissue, strain the liquid through a 400 mesh screen to collect the larvae. Back wash contents with tap water into a container. Add buffered formalin (see 13.1) to give a final concentration of 5% formalin.
- (e) Examine an aliquot for parasites. Alternatively, add washings to the contents from abomasum or small intestine for counting and identification.

#### 11.4.1. Digest Liquid

##### 11.4.1.1. Pepsin

Pepsin is available from BDH Chemicals Ltd, Poole, UK. The potency of different batches of pepsin varies: use 10 g of 3000 unit strength or 2 g of 150 000 unit strength.

Concentrated hydrochloric acid (30 mL) in 1 L water. A litre of digest liquid is sufficient to digest about 500 g of tissue.

### 11.5. Worm Counting

- (a) Process the abomasum and the small intestine separately. Adult worms from the large intestine can be counted macroscopically.

- (b) Dilute contents and digested remains to 2 or 4 L with tap water depending on the number of helminths present. Mix the contents thoroughly to obtain an even distribution of worms. Use compressed air and line. Adjust the rate of mixing so that none of the contents splashes out. Mixing should be in a criss-cross pattern not in a circular motion.
- (c) Remove a 10% aliquot and assay for parasites. The aliquot may be counted in one step or several subaliquots totalling 10%. Use 50 or 100 mL scoops for subsampling.
- (d) Stain each aliquot with parasitological iodine for few minutes. Decolourise with 20% sodium thiosulfate. Inhibited L<sub>4</sub> larvae will decolourise very quickly.
- (e) Examine under a dissecting microscope at x15 magnification using a petri dish marked with parallel lines 5 mm apart.
- (f) Count and differentiate the worms. The total number present in each organ is calculated from the dilution factors.
- (g) Storage solutions for helminths are listed in 13.1. Various techniques for being statistically accurate in the estimate of the number of worms present in an organ have been developed (Clarke and Turton, 1973; Reinecke, 1973).

### 11.6. Differential Worm Count

- (a) Identify to species and stage, the first 100 worms seen in each organ. Calculate the number of each species present and its stage as a per cent of the total.
- (b) Immature worms can be cleared in lactophenol (see 13.1.) for a few hours prior to identification under x100 magnification.

### 11.7. Worm Identification

The following descriptions are only intended to give a general idea of the genus present (Skerman and Hillard, 1966; Arundel, 1984). For detailed speciation see Soulby (1965). For immature stages refer to Douvres (1957).

#### 11.7.1. Abomasum

The three worms commonly found are *Haemonchus* sp., *Ostertagia* sp. and *Trichostrongylus axei*. They can be differentiated easily with the naked eye using length and thickness as the criteria.

*Haemonchus* sp. are large worms, up to about 25 mm long. The female is easily recognised by the characteristic barber's pole effect formed by the white ovaries wound spirally around the blood-filled intestine. The vulval flap can often be seen.

*Ostertagia* sp. are slender brown worms to about 12 mm, uniform in thickness throughout the length.

*T. axei* is very small, about 4–5 mm, and tapers markedly to the anterior end.

### 11.7.2. Small Intestine

The worms commonly present are *Trichostrongylus* spp. and *Nematodirus* spp. and these can be identified macroscopically on size and the marked tapering of *Trichostrongylus*.

*Trichostrongylus* spp. are small, slender, strongly tapering worms, about 11 mm.

*Nematodirus* spp. are much longer, the female reaching a length of about 23 mm. The characteristic filiform anterior end is usually tightly coiled. The male is much smaller, 10–15 mm long and is often coiled. It rarely stains as deeply as the other worms present and care must be taken not to confuse it with *Trichostrongylus* spp.

*Cooperia* spp. rarely occur in large numbers in sheep, but are common in cattle. They are reddish in colour and are larger, thicker and more uniform in thickness than *Trichostrongylus* spp. and are usually found in a flat coil. The male bursa is obvious to the naked eye.

*Strongyloides papillosus* is occasionally seen in large numbers. They are small parasites reaching about 6 mm. They never stain well with iodine and care must be taken to differentiate them from immature forms of *Trichostrongylus* spp. and *Nematodirus* spp.

Microscopically the oesophagus of *S. papillosus* is about one-third of the length of the worm, while the 4th larval stage of *Nematodirus* has a spine on the blunt tail.

### 11.7.3. Large Intestine

*Trichuris ovis* and *Oesophagostomum venulosum* are seen in the caecum of the sheep and can be differentiated easily on the characteristic whip worm morphology of *Trichuris*.

*Chabertia ovina* and *Oe. columbianum* (sheep) are mainly found in the colon but in heavily infested animals *Oesophagostomum* spp. may be found also in the caecum. *C. ovina* can be readily identified by the large buccal capsule while the *Oesophagostomum* spp. taper at both ends.

## 12. References

- Anonymous (1989). Anthelmintic Resistance: Report of the Working Party for the Animal Health Committee of the Standing Committee on Agriculture. (SCA Technical Report Series, No. 28). (CSIRO for the Australian Agricultural Council Standing Committee on Agriculture: East Melbourne, Victoria.)
- Arundel, J.H. (1984). 'Laboratory Manual for Veterinary Parasitology', School of Veterinary Science, University of Melbourne.
- Baldock, F.C., Lyndal-Murphy, M., and Pearse, B. (1990). An assessment of a composite sampling method for counting strongyle eggs in sheep faeces. *Australian Veterinary Journal* 67, 165–7.
- Beck, T., Moir, B., and Meppem, T. (1985). The cost of parasites to the Australian sheep industry. *Quarterly Review of the Rural Economy* 7, 336–43.
- Clark, C.J., and Turton, J.A. (1973). Estimating roundworm burden and group sizes in anthelmintic trials with sheep and cattle. *Experimental Parasitology* 34, 69–75.
- Davies, R.G. (1971). Three special-purpose programs. In 'Computer Programming in Quantitative Biology'. Chapter 14, pp 410–21. (Academic Press: London.)
- Dikmans, G., and Andrews, J.S. (1933). Infective Nematode Larvae of Sheep and Goats. *Transactions of the American Microscopical Society* 52, 1–25.
- Douvres, F.W. (1957). Key to the identification and differentiation of the immature parasitic stages of gastrointestinal nematodes of cattle. *American Journal of Veterinary Research* 18, 81–5.
- Finney, D.J. (1971). 'Probit Analysis'. 3rd Edn. (Cambridge University Press: Cambridge.)
- Lacey, E., Redwin, J.M., Gill, J.H., Demargheriti, V.M., and Waller, P.J. (1990). A larval development assay for the simultaneous detection of broad spectrum anthelmintic resistance. In 'Resistance of Parasites to Antiparasitic Drugs'. (Eds J.C. Boray, P.J. Martin and R.P. Roush.) VIlth International Congress of Parasitology. MSD AgVet 1990.
- Le Jambre, L.F. (1976). Egg hatch as an *in vitro* assay of thiabendazole resistance in nematodes. *Veterinary Parasitology* 2, 385–91.
- Martin, P.J., and Wursthorn, L. (1990). RESO, CSIRO Animal Health Research Lab., Private Bag No. 1, Parkville, Victoria 3052.
- Ministry of Agriculture, Fisheries and Food (1977). 'Manual of Veterinary Parasitological Laboratory Techniques'. Technical Bulletin No. 18, Ministry of Agriculture, Fisheries and Food. (Her Majesty's Stationery Office: London.)
- Powers, K.G., Wood, I.B., Eckert, J., Gibson, T., and Smith, H.J. (1982). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine and ovine). *Veterinary Parasitology* 10, 265–84.
- Reinecke, R.K. (1973). The larval anthelmintic test in ruminants. Technical Communication No. 106, Department of Agricultural Technical Services, Republic of South Africa 106, 1–20.
- Skerman, K.D., and Hillard, J.J. (1966). 'A Handbook for Studies of Helminth Parasites of Ruminants.' Near East Animal Health Institute Handbook No. 2. (Food and Agriculture Organisation: Rome.)
- Soulsby, E.J.L. (1965). Textbook of Veterinary Clinical Parasitology. Volume 1. Helminths. (Blackwell Scientific Publications: Oxford.)
- Waller, P.J. (1986). Anthelmintic resistance in nematode parasites of sheep. In 'Agricultural Zoology Reviews'. Vol. 1. (Ed. G.E. Russel.) 3rd Edn. pp. 333–73. (Intercept Porteland: Newcastle upon Tyne, England.)
- Whitlock, H.V. (1948). Some modifications of the McMaster helminth egg counting technique and apparatus. *Journal of the Council for Scientific and Industrial Research in Australia* 21, 177–80.

## 13. Appendixes

### 13.1. Appendix I — Preparation of Reagents

#### 13.1.1. Embalming Fluid

Embalming Fluid (EF) is used to preserve gastrointestinal tracts submitted for total worm counts.

EF is a 20% aqueous solution of ethanol ( $C_2H_5OH$ ) with a little formaldehyde, lysol (see 13.1.7.) and glycerol ( $CH_2OH.CHOH.CH_2OH$ ) added. It will preserve small ruminant gastrointestinal tracts for at least two weeks. Formalin is unsuitable for this purpose as it makes the tract stiff and worm recovery very difficult.

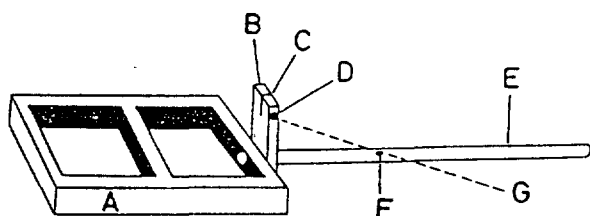
To make 20 L of EF:

Concentrated formalin	400 mL
Lysol	400 mL
Glycerol	1200 mL
95% Ethanol (absolute alcohol)	4 L
Tap water	14 L

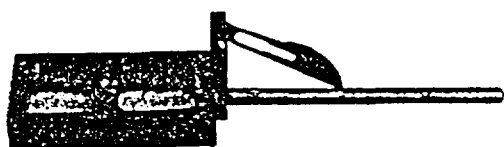
Shake before use.

## 13.1.1.1. Removal and preservation of small ruminant gastro-intestinal tracts for total worm counts

- Open carcase and locate various organs of the gastro-intestinal tract.
- Tie off (with string) abomasum at junction with omasum and pylorus. Sever connection with omasum. Collect faeces into a 25 mL bottle. Tie off rectum and sever at pelvic inlet.
- Free mesenteric attachment at the root of the mesentery and remove entire closed tract from carcase. Trim as much omentum as possible from tract.



(a)



(b)

**Figure 3.** (a) The 'gut' runner and (b) the 'gut runner' with blade in position.

Material: Brass or other non-corrosive material. Letters next to text refer to labels on Fig. 3a.

- Base framework. Length 8 cm, width 5 cm. Framework pieces. Height 13 mm, width 6 mm. The lower edges of the framework must be squared to provide scraping edges.
- Blade carrier, centrally placed at one end of base framework. Overall height 4 cm (top to bottom of base framework). Width 1 cm. Thickness 8 mm. The base framework and blade carrier can be assembled from separate pieces, or cast as one piece.
- Blade groove, cut centrally into top of blade carrier 1.5 cm deep, 0.5 mm wide.
- Locking screw; a threaded brass screw is embedded into the side of the blade carrier to secure the blade in position when inserted in the blade groove.
- Guide rod, a solid cylindrical brass rod, diameter 7 mm, length 12 cm, one end embedded and welded into the base framework, passing centrally through the blade carrier.
- Blade-tip anchor hole. This is small hole 1 mm diameter about 2 mm deep drilled centrally on the top edge of the guide rod in line with the blade groove and 4.4 cm from the blade carrier. It secures the point of a removable scalpel blade (Swann-Morton 22 A). It is drilled at a suitable angle to receive the tip of the blade.
- Angle of inclination for blade-tip anchor hole, taken from the bottom of the blade groove through the anchor hole position.

- Inject 20 mL EF into each of the abomasum, caecum and colon. Knead organs to mix EF throughout contents.
- Place tract in heavy duty plastic bag (450 x 300 mm, good for small sheep) and add 300 mL EF. Swirl fluid to ensure good contact with tract. Seal bag by tying a knot, excluding as much air as possible. Place this bag in a second bag for extra security against spillage and seal similarly.
- Preserved tracts held at room temperature will be suitable for total worm counts for at least two weeks.
- Transport in an esky or other rigid container.

## 13.1.2. Lactophenol

Lactic acid, $\text{CH}_3\text{CHOHCOOH}$	1 part
Phenol, $\text{C}_6\text{H}_5\text{OH}$	1 part
Glycerine	1 part
Distilled water	1 part

- Mix ingredients.
- Place specimen to be cleared on microscope slide, cover with lactophenol and warm gently until fumes start to rise.
- Add more lactophenol if necessary, cover-slip and examine.

*N.B.* Lactophenol will cause some shrinkage.

## 13.1.3. Flotation Solutions

### 13.1.3.1. Sodium chloride, saturated solution specific gravity of 1.20

- Dissolve commercial grade salt (superfine kiln dried) in almost boiling water until no more dissolves.
- Prepare a stock supply so that at least one-quarter of the volume is undissolved salt.
- Stir the solution for 30 min before use to ensure a saturated product.
- Filter the solution to remove the debris.
- Measure the specific gravity with a hydrometer or weigh; 100 mL should weigh 120 g.

### 13.1.3.2. Magnesium sulfate, saturated solution specific gravity 1.3

This solution can be maintained at maximum specific gravity in a range of ambient temperatures, 25°C.

## 13.1.4. Parasitological Iodine

Iodine, $\text{I}_2$	30 g
Potassium iodide, KI	40 g
Water to	100 mL

Dissolve potassium iodide in water, then add iodine crystals. *N.B.* This is a strong aqueous solution so that only a few drops are needed.

## 13.1.5. Storage Solutions for Helminths

### 13.1.5.1. Alcohol

70% Alcohol (ethanol)	95 parts
Glycerine	5 parts

### 13.1.5.2. Formalin, 5% solution

Commercial formalin, (40 % formaldehyde)	5 parts
Water	95 parts



**Table 8.** Anthelmintics for the control of gastro-intestinal nematodes of sheep

Brand name	Chemical ingredient	Supplier
<b>Broad spectrum drenches</b>		
<b>I — Benzimidazoles Group (BZ)</b>		
Thiabendazole	Thiabendazole	Merck Sharp & Dohme
Ranizole	Thiabendazole plus Rafoxanide	Merck Sharp & Dohme
Telmin WSD Mebendazole DHA Rural Mebendazole Panacur Valbazen	Mebendazole    Fenbendazole Albendazole	SmithKline, Beecham    Hoechst Australia SmithKline, Beecham
Closal	Albendazole plus Closantel	SmithKline, Beecham
Systemex	Oxfendazole	Coopers
Synanthic		Syntex
Rycoben	Ricobendazole	Youngs
Rintal	Febantel	Bayer
Elderado Armadrench		
<b>II — Levamisole/Morantel Group</b>		
Nilverm		Coopers
Ripercol	Levamisole	SmithKline, Beecham
Citarin	Levamisole	Bayer
Levamisole oral drench	Levamisole	
National Levamisole		
WSD Levamisole		
Elderado Levamisole		
Levi Gold		
Nilverm Spot M	Levamisole	Coopers
Nilvax	Levamisole (Injectible) plus 5 in 1 Clostridial vaccine	Coopers
Nilzan	Levamisole plus Oxytetracycline	Coopers
Exhelm-E Oralject	Morantel	Pfizer
<b>III — Combination Group</b>		
Combi	Ricobendazole/Levamisole	Youngs
Salvo	Fenbendazole/Levamisole	Hoechst
Scanda	Oxfendazole/Levamisole	Coopers
Specnel	Oxfendazole/Levamisole	Syntex
<b>IV — Ivermectin Group</b>		
Ivomec	Ivermectin	Merck Sharpe & Dohme
<b>V — Organophosphate Group</b>		
Rametin	Naphthaphos	Bayer
<b>VI — Capsule Group</b>		
Extender 100	Albendazole	Nufarm
Proftril	Albendazole	SmithKline, Beecham
<b>Narrow Spectrum Drenches</b>		
Seponver	Closantel	SmithKline, Beecham

13.1.6. Buffered Normal Formalin (20 L)  
 Disodium hydrogen phosphate, anhydrous  $\text{Na}_2\text{HPO}_4$  148 g  
 Sodium dihydrogen phosphate, dihydrate  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  101 g  
 Formalin (40%) 2 L  
 Water to make 20 L  
 Dissolve phosphates in hot water, cool and then add formalin and make up with water.

13.1.7. Lysol  
 Lysol is a brown, oily fluid, with antiseptic properties, made from coal tar by dissolving in fat and extraction with alcohol or by combining cresol with soap.

13.2. Appendix II — Equipment: The 'Gut-Runner' for Post-mortem Examination of Intestines (Skerman and Hillard, 1966)  
 (See Fig. 3 and following legend; 13.2.).

13.3. Appendix 3 — Anthelmintics for the Control of Gastrointestinal Nematodes of Sheep  
 See Table 8.