

Appendix 2

Standard operating procedures

A2.1. McMaster method of faecal worm egg count

Extracted from Van Schalkwyk, P.C., Schröder, J., Malan, F.S. and Van Wyk, J.A., 1995. Worm Workshop : Recommendations on Worm Control. Division of Helminthology, Onderstepoort Veterinary Institute, Pretoria, pp. 18-19. Modifications made in the studies included in this dissertation are indicated in square brackets.

A2.1.1. Purpose

The presence of worm eggs in the faeces provides evidence that an animal is infected. The McMaster method is a simple method for obtaining a count of the number of nematode eggs per gram of faeces. The advantage of the faecal worm egg count lies in the fact that when conducted routinely, the pattern of worm infection can be determined and the success (or failure) of parasite control can be monitored.

A2.1.2. Apparatus

A2.1.2.1. In the field

- a) Disposable plastic gloves.
- b) Plastic bags.
- c) Cooler box with ice pack.
- d) Pen and labels.
- e) Ear tags.

A2.1.2.2. In the laboratory

- a) Scale/balance : Accuracy of 0,1g is required. [Triple beam balance, Ohaus Scale Corporation, Florham Park, N.J., USA.]
- b) Microscope : A compound microscope capable of 80-100X magnification. The ideal combination is a 10X objective with 8X oculars (=80X). The field must be wide enough to cover one lane of the ruled grid of the McMaster slide.
- c) McMaster counting chambers (slides). [Eggs-ActoTM McMasters, E. Krecek, South Africa.]
- d) Blender/homogeniser (optional). [IKA[®] - Labortechnik, Janke and Kunkel, N.T. Laboratory Supplies, Johannesburg.]
- e) Measuring cylinder (100ml capacity) or a syringe (>60ml capacity). [A dispenser was used – ZippetteTM, Jencons Scientific, England.]
- f) Medical 100ml glass bottle with neck wide enough to fit over blender shaft.
- g) Pasteur pipettes with a suction bulb or alternatively, cooldrink or artificial insemination (AI) straws, to fill the McMaster slide. [LiquipettesTM, Elkay, Ireland or equivalent.]
- h) Glass beaker of ca. 500ml capacity for composite samples.
- i) Reagents : Amyl alcohol. [Substituted with polypropylene glycol (Polypropylene glycol P400TM, Fluka) in January 2000 when a technician showed symptoms of a hazardous chemical injury due to amyl alcohol.]

Sugar solution (40 %).

A2.1.3. Method

A2.1.3.1. Collection of faeces

Faeces should be collected from the rectum and not picked up from the ground. Smaller animals such as young lambs can usually be induced to defaecate by inserting a finger into the rectum and

gently massaging until the sphincter relaxes. Collect the pellets in a disposable glove or place in a plastic bag.

A2.1.3.2. *Individual counts*

- a) Weigh 2g (sheep) or 4g (cattle) of faeces and place in a 100ml glass bottle. Add 58ml (sheep) or 56ml (cattle) sugar solution and blend until the faeces is well dispersed (10 to 20 seconds).
- b) Add six to 10 drops of amyl alcohol to the mixture and invert to mix. Leave for two minutes to allow air bubbles to break.
- c) Wet the McMaster slide, shake well to remove excess water and wipe the outside dry with a paper towel. [This step was omitted.]
- d) Mix the sample again by inversion or by blowing air through it with a cooldrink straw or AI pipette. [Samples were vigorously mixed with a Pasteur pipette.] Withdraw fluid without delay and fill the McMaster slide.
- e) Fill two chambers of the McMaster slide and leave standing for at least two minutes before counting. Specimens should be examined within an hour after preparation.
- f) Calculate the eggs per gram of faeces by multiplying the total number of eggs counted in the two chambers by 100 for sheep and by 50 for cattle.

Example : First chamber : 6
 Second chamber : 8
 Total : 14

Eggs per gram = 14 x 100 = 1400 (sheep)

Eggs per gram = 14 x 50 = 700 (cattle)

A2.1.3.3. Composite samples

From 10 to 15 specimens collected individually, weigh out 1g from each and pool them. Add 29ml sugar solution for each gram and blend as described above. Add 15 drops of amyl alcohol and proceed with the count as described above for individual samples. Calculate the result by the method for sheep above. A more detailed description of preparing composite faecal samples appears elsewhere in Van Schalkwyk et al. (1995).

A2.2. Trematode egg count

Extracted from Van Wyk, J.A., Schröder, J., Van Schalkwyk, P.C. and Horak, J.G., 1987. Tegnieke : Helmintologie (in Afrikaans). In : Schröder, J. (Ed.), Proc. Worm Resistance Workshop. Pretoria, South Africa, pp. 125-127. Translated here into English. Modifications made in the studies included in this dissertation have been indicated in square brackets.

A2.2.1. Purpose/Description

In contrast with the eggs of gastrointestinal roundworms, trematode eggs have such a high specific gravity that undamaged eggs do not float in flotation fluid.

The presence of eggs in faeces is thus determined by concentrating the eggs by means of sieving and/or sedimentation. In the process, some of the finer, the larger, as well as the lighter faecal particles are discarded, so that the eggs may be more easily seen. Addition of methylene blue stain increases the contrast between the eggs and the faecal particles, without making the identification more difficult.

A2.2.2. Apparatus

- a) Helminth filter or sieves (64µm and 150µm).
- b) Funnel sieve (with a 150µm sieve in the funnel). [150µm (United wire test sieve, Nigel, South Africa or equivalent) and 38µm (Labotec test sieve, Johannesburg or equivalent) metal sieves.]
- c) 100ml [200ml] measuring cylinder.
- d) 10mP [20ml] pipette.
- e) Petri dish with parallel lines on the base. [70mm x 70mm gridded perspex container, E. Krecek, South Africa.]
- f) Dissection microscope.
- g) Methylene blue 1%.
- h) 2P fruit jar [3P utilized when 2P not available].
- i) Garden hose spray.

A2.2.3. Method

The technique that is used will depend on the available apparatus. For a quantitative diagnosis, the sample that is being concentrated for examination may be subdivided and only a portion of this examined.

A2.2.3.1. Helminth filter method

[This method was not utilised in the present study, but is included here since it is referred to in the sedimentation method described below.]

The apparatus consists of two sieves which fit into each other – the innermost one allows trematode eggs to pass through, and the outermost one does not allow eggs through, but is permeable for fine faecal particles.

The apparatus is relatively expensive, but will definitely be worth the trouble of purchasing if the practice is located in an area such as the Mpumalanga Province pan veld or the Free State Province, where trematode infection is common.

- a) Weigh off 5g of faeces.
- b) Place the faecal sample in the innermost filter.
- c) Set the hose spray to give a small rosette stream, and not a thin stream.
- d) Spray a strong stream of water over the faecal sample, until the water that flows out of the filter is clean.
- e) Tap the contents of the outermost filter into a 100ml measuring cylinder.
- f) Make up the cylinder contents to 100ml.
- g) Mix the sample by covering the mouth of the cylinder with your hand and quickly inverting the cylinder a couple of times. Immediately hereafter withdraw 10ml of the suspension and transfer this to a Petri dish.
- h) Examine the sample (aliquot) little by little in a gridded perspex Petri dish under a dissection microscope, at a magnification of approximately 25X. The contrast between the eggs and the faecal particles, which make up the background may be enhanced by adding 1% methylene blue to the sieved sample and allowing this to stand for five minutes before it is washed on a 38µm sieve and the material on the sieve collected for examination. [Methylene blue was not utilized in the current study.]
- i) $\text{Count} \times 2 = \text{worm egg count per gram of faeces.}$

Practical hints

- a) Trematode egg counts are more time-consuming to carry out than those of roundworms. Try, therefore, first to establish whether the farm from which the samples originate poses a real danger of trematode infection (vleis or dams) before deciding to carry out a trematode egg count. Remember, however, that the animals may have been brought in from elsewhere, and that liver fluke infection may sometimes arise in drinking troughs.
- b) The hose spray (which should preferably be connected with an instant clip-on attachment to the tap) is most effective when it is adjusted to provide a small rosette spray, instead of a thin spray, which tends to cause the sample to splash out of the sieve, and thus cause a falsely low count.
- c) Move the spray continuously up and down in the innermost filter while the sample is being filtered, to spread out the whole wall of this filter, and to mash through the eggs.
- d) The eggs of *Fasciola* spp. are oval, like those of *Paramphistomum* [*Calicophoron*], but are yellowish in colour, in contrast with the colourless transparent eggs of the latter. Sometimes cases occur where the eggs of *Paramphistomum* are light brown, but their appearance is more granular than those of *Fasciola* spp. The eggs of both types have a relatively clear operculum at one of the poles, and are undeveloped in fresh faeces – in other words, they do not contain a developed miracidium. In contrast, the eggs of *Schistosoma mattheei* are spindle-shaped, there is no operculum, and they contain a developed miracidium.
- e) It is recommended that a reference sample of, for example, *Fasciola* eggs be kept to compare with samples from the field. The eggs can, for example, easily be obtained by washing the gall bladder contents of an infected animal on to a 38µm sieve.
- f) The eggs of *S. mattheei* hatch quickly in water, and formalin must therefore be added before the faeces are processed to prevent hatching while the faeces are washed on the sieves or in the filter. These eggs sediment relatively slowly, and as a result the jar must be left to stand for up to 15 minutes during concentration of the eggs (see below) before the water is discarded.

A2.2.3.2. *Sedimentation method*

[Modified as described in Chapter 2 for use in the present study.]

Through repeated dilution of the faecal suspension, and sedimentation of the eggs (which are heavier than most of the faecal particles), the faeces in the sample are reduced and the eggs concentrated so that they are more easily observed. The process can be made very much easier if the faeces are initially sieved through a 150µm sieve.

- a) Weigh off 5g of faeces, and crush these finely.
- b) If possible, sieve with water through a 150µm funnel sieve, which is placed above a container to collect the filtrate, which passes through the sieve. [In the present study, the faeces were sieved through a 150µm metal sieve (United wire test sieve, Nigel, South Africa or equivalent) into a 38µm metal sieve (Labotec test sieve, Johannesburg or equivalent), using water sprayed from a nozzle at high pressure. The sediment remaining on the 38µm sieve was washed into a 2 or 3P fruit jar.]
- c) Place the finely crushed faeces or the filtrate from the 150µm sieve into a 2P fruit jar, and fill with water.
- d) Allow to stand for four minutes (longer for *Schistosoma* – see above) so that the eggs sink to the bottom of the jar. [In the present study, the samples were allowed to stand for at least 15 minutes.]
- e) Pour off as much of the supernatant as possible, without losing any of the sediment.
- f) Repeat this process of sedimentation and discarding of the supernatant several times until the supernatant is clear within a few moments of the jar having been refilled.
- g) Discard the supernatant a last time after a standing time of four minutes, and pour the sediment into the measuring cylinder.

- h) Mix, take an aliquot, and examine microscopically as described for the filter method.

Practical hints

- a) More often than not the sample is ready for examination after it has been sedimented and decanted three times.
- b) If the bottle stands for too long after water has been added, too much of the faeces sediments out, and the eggs are not effectively concentrated. On the other hand, sedimentation for less than four minutes will cause eggs to be discarded before they have reached the base of the jar.
- c) Use of a water vacuum pump (a cheap apparatus which is fitted to a water tap) to remove the supernatant, will speed up the process of sedimentation, since a larger proportion of the supernatant may be removed each time, and together with it also a larger proportion of the faecal particles.

A2.3. Faecal larval culture

Extracted from Van Wyk, J.A., Schröder, J., Van Schalkwyk, P.C. and Horak, J.G., 1987. Tegnieke : Helmintologie (in Afrikaans). In : Schröder, J. (Ed.), Proc. Worm Resistance Workshop. Pretoria, South Africa, pp. 123-125. Translated here into English. Modifications made in the studies included in this dissertation have been indicated in square brackets.

A2.3.1. Purpose/Description

The *eggs* of the majority of the most common gastrointestinal roundworms differ morphologically so little from each other that they cannot be differentiated microscopically from each

other. Consequently, with a few exceptions, it cannot be determined from a faecal egg count which worm types are involved.

In contrast, the *infective larvae* of these worm types may be differentiated to genus level, and faecal cultures may thus be used in the live animal to determine with which worm types the animals are infected. It is certainly not easy to differentiate between the larvae, but the techniques to recover larvae are described here so that larvae may be recovered and sent to an expert for identification. It is hoped that this may stimulate more veterinary practitioners to learn the identification of larvae so that they may provide a better service to their clients.

Faeces are mixed with a medium which keeps them moist and aerated so that worm eggs may hatch and the resulting larvae may develop to the third stage. For the collection of the larvae, use is made of their instinctive tendency to migrate upwards in the presence of moisture and light, away from the faecal culture in which they hatch.

Two methods are described – the first for the collection of relatively small number of larvae (only for diagnostic purposes), and the other for the collection of larger numbers, e.g. for the infection of animals to enable the identification of the worm types to species level.

A2.3.2. Apparatus

A2.3.2.1. Diagnostic method (small numbers of larvae)

- a) 1 small wide-mouthed bottle of heavy glass, \pm 3cm in diameter x 2cm high.
- b) 1 larger wide-mouthed bottle, \pm 5cm in diameter x 4-5cm high.

A2.3.2.2 Collection of large numbers of larvae

- a) 1P fruit jar.
- b) 2 wooden dowelling rods, approximately 3cm in diameter x 40cm long.

- c) Plastic flat-bottomed mixing bowl, approximately 50cm x 30cm.
- d) McMaster canvas bags for collection of the faeces [not utilized].

A2.3.2.3. Both methods

- a) Vermiculite, or other culture medium, such as newspaper.
- b) Plastic washbottle.
- c) 100ml measuring cylinder.
- d) Counting container with lines on the base of the container.
- e) Standard microscope.
- f) Stereo dissection microscope.
- g) Pasteur pipette with suction bulb.
- h) Glass slides.
- i) Cover slips (22mm x 40mm).
- j) Lugol's iodine.

A2.3.3. Method

A2.3.3.1. Diagnostic larval culture

[This method was not utilised in the present study, but is included here since it is referred to in the section on "Collection of large numbers of larvae" described below.]

- a) Collect the faeces from the animal as for a worm egg count.
- b) Sample approximately 10g of faeces.
- c) Break the sheep faeces into fine pieces by flattening the pellets.
- d) Mix cattle dung or unformed sheep faeces with vermiculite (approximately equal amounts of each), and moisten the mixture without it becoming soft.

- e) Fill the small bottle (3cm x 2cm high) level with the faeces mixture.
- f) Place the small bottle in the larger one, and add water to the larger bottle up to the brim of the small one.
- g) Screw on the lid of the larger bottle and incubate the sample for seven to 10 days at $\pm 27^{\circ}\text{C}$ (Reinecke, 1983).
- h) Thereafter remove the smaller bottle with a tissue forceps and pour the fluid from the larger bottle into a test tube (Reinecke, 1983).
- i) Allow the tube to stand for 20 minutes [15 minutes or longer in the present study] so that the larvae settle and with the help of a Pasteur pipette [LiquipettesTM, Elkay, Ireland or equivalent] carry a few drops of the sediment over to a glass slide (Reinecke, 1983).
- j) Add a drop of iodine, cover with a cover slip, and identify the larvae ($\pm 10\times$ objective lens).

Practical hints

- a) As with all similar samples, labelling and identification of the samples is essential.
- b) Dry, sterilised faeces, charcoal or even old newspaper may be used in the place of vermiculite. The aim of the substance is to aerate wet faeces, otherwise the worm eggs will not hatch.
- c) If there are too little faeces to fill the small bottle, cotton wool may be placed in the bottom of the bottle and wetted before the faeces are placed on top of it (Reinecke, 1983).
- d) In the case of cattle faeces, rather make two cultures since there are often few eggs, and hence larvae, in the faeces (Reinecke, 1983).
- e) Should the identification of the larvae take a long time, or if this cannot be done immediately, evaporation can be prevented by smearing petroleum jelly on the edges of the cover slip before it is placed on the drop of larval suspension on the slide (Reinecke, 1983).

A2.3.3.2. Collection of large numbers of larvae

[Used in the present study.]

- a) Place a McMaster faecal bag on the sheep or bovine to collect sufficient faeces.
- b) Break the sheep faeces into fine pieces.
- c) Mix the faeces with vermiculite (approximately equal amounts of each).
- d) Hold one of the wooden dowelling rods in the middle of the fruit jar while the faecal mixture is placed little by little in the bottle and pressed lightly down around this rod with the second rod up to a maximum height of 5-7,5cm (Reinecke, 1983).
- e) With a tissue, wipe off excess faeces on the inside of the jar (Reinecke, 1983). If this is not done, the larval suspension may be contaminated with faeces and pieces of vermiculite during harvesting of the larvae.
- f) Rinse the inside of the fruit jar with the washbottle down to the surface of the compacted faeces.
- g) Adjust the moisture content until it is damp but not too soft.
- h) Screw on the lid of the flask lightly and incubate for seven to 10 days at 27-30°C (Reinecke, 1983).
- i) Thereafter flush the inside of the flask down to the surface of the culture and place this in indirect sunlight in the laboratory (Reinecke, 1983). [This step was omitted in the present study.]
- j) After one to two hours the larvae migrate up the sides of the flask. Collect the larvae by holding the flask upside down and by flushing the larvae off the sides and allowing these to run into a 100ml measuring cylinder.
- k) Allow the larvae to sediment out and examine a sample thereof as for the diagnostic method.

Practical hints

- a) Ensure that the dowelling rods are thoroughly cleaned between faecal samples, otherwise cross-contamination may occur.

- b) Sheep faeces may be crushed finely by stamping the faeces lightly before they are removed from the bag.
- c) The faecal mixture in the flask is only compacted enough so that it withstands handling without crumbling during collection of the larvae.
- d) Often only a portion of the larvae in the larval culture migrate out of the culture initially with the first flushing and the flushing and collection may be repeated a couple of times to collect more larvae.
- e) *Nematodirus* eggs do not hatch within seven days in the culture. The culture may be left for 14 days, but then fungal growth may create problems. Another method must thus usually be used for the collection of these eggs.
- f) The eggs of certain worm types, such as those of *Trichuris* and *Toxocara*, do not hatch in the cultures. These worm types can however be identified from the egg morphology to the genus level.
- g) Hookworm larvae (*Bunostomum*, *Gaigeria* and *Ancylostoma*) sometimes apparently do not migrate upwards out of the culture. In such a case, the larvae may be collected by filling the jar with water and by inverting it as follows : fill the jar until the water meniscus bulges above the edge of the jar, place a large Petri dish upside down on the jar, and turn the jar and dish upside down so that the fruit jar stands upside down in the Petri dish. Now add water to the Petri dish to a depth of 2-3cm. Slide a microscope slide under the lip of the fruit jar to leave a small space between the bottom of the dish and the edge of the flask. After one to two hours the water is collected from the Petri dish and worm larvae are to be found therein (Reinecke, 1983).

A2.4. Identification of infective third-stage nematode larvae of small stock and cattle

The keys described here are those referred to in Van Wyk, J.A., Alves, R.M.R. and Michael, L.M., 1997a. A novel key for identifying nematode infective larvae (L₃) from domesticated ruminants. In : Proc. 16th International Conference of the World Association for the Advancement of Veterinary Parasitology, Sun City, South Africa.

The easiest way to identify infective L₃ of small stock and cattle is to compare the lengths of the free sheaths (sheath tail from the posterior tip of the tail to the tip of the sheath) of different genera with one another.

For instance, measure the length of the sheath tail of *Trichostrongylus colubriformis* L₃ with the aid of a compound microscope graticule and at a 100X magnification, and let this equal "X". Then the free sheath tail of *Haemonchus contortus* is 2"X".

The following tables provide keys to the identification of the more common L₃ in South Africa.

Please note, however, that the measurements of some helminth field strains differ somewhat from the tables (e.g. *H. contortus* with a free sheath tail shorter than 2"X"). Remember also that only the measurements of the most common worm species are included; the tail sheaths of *Trichostrongylus falculatus* L₃ are, for instance, somewhat longer than those of *T. colubriformis*.

It remains difficult to identify nematode larvae, and this manual is only intended as a guide after a person has already had practical training. In other words, it is unlikely that someone without practical training will be able to identify larvae accurately using this manual. Furthermore, even the most experienced person may become confused after long sessions of larval identification and become hesitant about even the most common worm species. The best advice in this case is to stop identifying

the larvae until one's mind is fresh again. It is also essential to have a standard set of L₃ of pure helminth strains, so that comparisons can be made when larvae in field samples are difficult to identify.

It is most important particularly in the period immediately after a practical training course, to practise larvae identification or else it may be difficult to perceive the subtle differences between the various larvae.

Preparing larvae for identification

Transfer a small aliquot of larvae (harvested from faecal cultures) using a Pasteur pipette, to a microscope slide, add a drop of iodine and cover with a coverslip. Examine microscopically using the following keys.

Table A2.4.1

A key for the identification of nematode third-stage larvae of small stock

No.	Description	Identification
1.	Sheath tail absent	2
	Sheath tail present	3
2.	Sheath tail absent	
2.1	Oesophagus \pm 40% of total length of larva; without a bulb; larva very thin; "head"* relatively bullet-shaped; tail appears blunt, like a sheath tail that has broken off. <i>Examine carefully: it often appears as if the larva does have a sheath</i>	<i>Strongyloides</i>
2.2	Oesophagus < 40% of length of larva and rhabditiform (double bulb); thick, cigar-shaped larva with a long tail that may appear to have a sheath; tip of "head" has a definite "structure" and is reasonably flattened	Free-living nematode
3.	Sheath tail present; oesophagus < 40% of total length of larva	
3.1	Oesophagus with a single bulb; larva stains \pm uniformly with iodine	5.3 + 8
3.2	Oesophagus without a bulb; larva stains light anteriorly and dark posteriorly with iodine	4
4.		
4.1	Sheath tail invisible; larva lethargic and has a protuberance on the "head"	<i>Dictyocaulus</i>
4.2	Sheath tail visible; fresh larva very active; no protuberance on the "head"	5
5.	Sheath tail length	
5.1	Short, without filament	6
5.2	Medium, with short filament	7
5.3	Medium, with long filament	8, 9
5.4	Long, with long filament	10,11
6.	Length of sheath tail "X"; head somewhat flattened; sheath tail narrows fast to a point (resembles a pencil point); no filament	
6.1	Larva tail smooth when exsheathed	<i>Ostertagia/Teladorsagia</i> , <i>T. axei</i> and <i>Marshallagia</i>
6.2	Exsheathed larva tail unevenly serrated (digitate)	Intestinal <i>Trichostrongylus</i>
7.	Length of sheath tail 2"X"; "head" \pm bullet-shaped and tapers relatively fast to a point (\pm slanted sides); sheath filament short (\pm 15% of sheath tail length)	<i>Haemonchus</i>

No.	Description	Identification
8.	Length of sheath tail 3“X”; short larva which stains uniformly with iodine; “head” bullet-shaped; sheath filament long (\pm 50% of sheath tail length); oesophagus with single bulb	<i>Gaigeria</i>
9.	Length of sheath tail 3“X”; “head” broad and flattened; sheath filament long (\pm 25% of sheath tail length); 28-32 intestinal cells (rectangular shape) in <i>fresh</i> larva	<i>Chabertia</i>
10.	Length of sheath tail 4,5“X”; “head” broad and flattened (with nearly parallel sides); 18-22 intestinal cells (triangular shape) in <i>fresh</i> larva; sheath filament long (\pm 50% of sheath tail length)	<i>Oesophagostomum</i>
11.	Length of sheath tail 8“X”; “head” is bullet-shaped but somewhat flattened; sheath filament is very long (\pm 50% of free sheath length); larva tail with finger-like projection (only clearly visible in exsheathed larva)	<i>Nematodirus</i>

* “Head” refers to the cranial/anterior tip of the larva.

Bear in mind that the L₃ of many genera look as though the “head” is flattened when the larvae are partially or totally exsheathed.