Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: A practical lab guide

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Dates:
Received: 19 Nov. 2012
Accepted: 20 Dec. 2012
Published: 13 Mar. 2013

How to cite this article:
Van Wyk, J.A. & Mayhew, E., 2013, 'Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: A practical lab guide', *Onderstepoort Journal of Veterinary Research* 80(1), Art. #539, 14 pages. http://dx.doi.org/10.4102/ojvr.v80i1.539

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In 2004, a new concept was introduced for simplifying identification of larvae of the common nematodes of cattle, sheep and goats that comprises estimates of the lengths of the sheath tail extensions of infective third-stage larvae (L_3) of each genus and/or species to that of *Trichostrongylus* spp., instead of having to be dependent only on measurements in micrometre. For example, if the mean length of the sheath tail extension (the extension of the sheath caudad, beyond the caudal tip of the larva) of *Trichostrongylus colubriformis* and *Trichostrongylus axei* is assumed to be 'X', then that of *Haemonchus contortus* is 2.0–2.7 'X' – a difference that is not difficult to estimate. An additional new approach suggested now, particularly for L_3 of species and/or genera difficult to differentiate (such as *Chabertia ovina* and *Oesophagostomum columbianum*), is to estimate the proportion of the larval sheath tail extension comprising a terminal thin, whip-like filament. For the experienced person, it is seldom necessary to measure more than one or two sheath tail extensions of L_3 in a mixed culture, because the identity of most of the remaining L_3 can thereafter be estimated in relation to those measured, without having to take further measurements. The aim of this article was to present the novel approach in the form of a working guide for routine use in the laboratory. To facilitate identification, figures and a separate organogram for each of small ruminants and cattle have been added to illustrate the distinguishing features of the common L_3.

Introduction

Based on a review by Van Wyk, Cabaret and Michael (2004) and helminthological literature over nine decades, the present article is aimed, through a novel approach and illustrations drawn to scale, at facilitating the morphological identification of infective nematode larvae (L_3) of the common nematodes of small ruminants and cattle.

Diagnosis of parasitic nematode infections of ruminants, both qualitative and quantitative, is largely still dependent on relatively inaccurate methods such as faecal worm egg counts and accompanying larva identification, without which no indication can be obtained of the identities of most of the common worm genera, excepting for those genera with morphologically distinct ova, for example *Strongyloides papillosus*, *Nematodirus* spp. and *Trichuris* spp. In contrast, ova of *Ostertagia*, *Teladorsagia*, *Trichostrongylus*, *Oesophagostomum*, *Chabertia* spp. and, to some extent, *Cooperia*, *Bunostomum* and *Gaigeria* spp. are either difficult or impossible to differentiate without measurements and computations that are, as yet, impractical for routine use. Although some progress has been made with computerised identification (Christie & Jackson 1982), this has not been developed for general use.

Large differences in the pathogenicity of the common worm genera make it essential to know which nematode genera are responsible for cases of morbidity of animals. At present, the only practical method available for routine laboratory estimation of the proportions of the worm genera present in the living animal, is to identify the larvae that are found in fresh faeces (mostly lungworm larvae) or those that develop in faecal cultures (gastrointestinal nematodes). However, it is often only the experienced person who can identify the larvae with a high degree of accuracy and few such persons remain for training the inexperienced. For many of the nematode genera, distinguishing features such as the shape of the cranial extremity (the head) of the larva are practically indistinguishable to all but the practised eye. Measuring first-stage larvae (L_1) of protostrongylids and the L_3 of strongyles, or the length of the sheath tail extension (STE) (from the caudal tip of the larva to the tip of the STE – 'c' in Figure 1) can aid identification, but, being too time-consuming, it is not practical to measure each larva during routine differential diagnosis.

In this article, a simplified, better-structured method is described for differentiating the L_1 of various genera of protostrongylids and the L_3 of strongyles from one another. Whilst some

conventional characteristics are still used for identification, the novel approach principally comprises a comparison of the lengths of the STEs of L_3 of the unknown identity to those of *Trichostrongylus axei* and *Trichostrongylus colubriformis* and estimating the proportion (if any) which comprises a whip-like filament ('d' in Figure 1).

Materials and methods

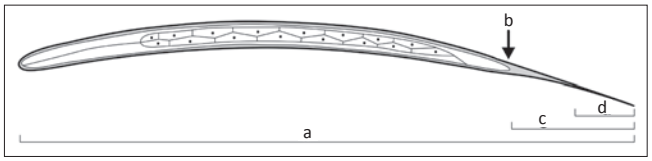
Only some methods which are not more or less universal laboratory proceedings are included below.

Faecal cultures and harvesting of infective larvae

There are numerous different methods for preparing faecal cultures. However, in this laboratory they are prepared as follows by a modification of the technique of Roberts and O'Sullivan (1950), as described by Reinecke (1973) for all but the various lungworm and *Nematodirus* species. In short, sheep faecal pellets are thoroughly crumbled before being mixed with sufficient vermiculite chips to yield a crumbly mixture which is lightly compacted, using non-porous stampers, to a depth of about 5 cm in wide-mouthed glass jars of approximately 1 L capacity. A hole is left in the centre of the culture by holding a stamper vertically in the centre of the jar whilst the mixture is lightly compacted around it. The culture is moistened sufficiently to ensure that it does not dry out whilst being incubated, but without it becoming water-logged. Thereafter, the jar is incubated in the dark at 26 °C – 28 °C for 5–7 days, during which time it is checked periodically and moistened if necessary. Note that, as discussed by Van Wyk *et al*. (2004), the length of both the larva and its STE may vary in relation to the amount of moisture in the culture medium (Rossanigo & Gruner 1996), so this needs to be standardised for consistent results.

After the 7-day period of incubation of the culture, the inside of the jar is sprayed lightly with water from a wash bottle before being placed in bright light that stimulates the L_3 to migrate up the inner surfaces of the vessel's walls. The culture is harvested repeatedly over several days by holding the jar at a slant with the mouth pointing downwards and then spraying the inner walls with a wash bottle and allowing the larval suspension to drain into suitable containers.

As *Gaigeria pachyscelis* and *Bunostomum phlebotomum* do not migrate readily up the walls of the culture jars, they are harvested by filling the culture jar with water, allowing it to stand for a few minutes to allow the air to escape from the culture, adding water to the jar until the water meniscus protrudes above the lip of the jar, placing an overturned Petri dish over the mouth of the jar and keeping the Petri dish in position whilst the jar is inverted (Borgsteede & Hendriks 1974; Eckert 1960). Water is then added to the Petri dish and the rim of the jar is lifted slightly from the bottom of the dish on one side by slipping two glass microscope slides under it. The preparation is left for a few hours for L_3 to migrate into the water and to settle, before the water in the Petri dish is removed with a pipette for larval identification and counting.



Source: Drawn from Borgsteede, F.H.M. & Hendriks, J., 1974, 'Identification of infective larvae of gastrointestinal nematodes in cattle', *Tijdschrift Diergeneeskunde* 99, 103–113.

FIGURE 1: Diagram of a nematode infective larva, depicting (a) total length, (b) tip of larva tail, (c) sheath tail extension and (d) filament.

Because *Nematodirus* spp. ova need up to 14 days to hatch, fungal overgrowth commonly makes the above culture method unsuitable for members of this genus. Thus the ova are initially concentrated in relatively little faecal suspension by sieving through a combination of sieves with apertures of different sizes. So-called Visser sieves (a set consisting of three tubular sieves fitting one into the other) (Malan & Visser 1993) are ideal, with sieve openings of about 200 µm in the inner tube, 150 µm in the middle and 38 µm – 60 µm apertures in the outer tube sieve. The latter sieve is capped at the bottom to accumulate the contents in about 20 mL of water, whilst a tap is provided in the cap for drainage. The faecal sample is placed in the inner tube sieve before being thoroughly washed with a rosette of spray using an adjustable garden spray on a hose, with the effluent passing through all three tubes. The inner tube sieve is then removed and the contents of the middle tube washed similarly before it is removed in turn and the process repeated for the outer tube. *Nematodirus* spp. and *Marshallagia marshalli* ova are retained in suspension in the inner tube and are cleaned further by progressive removal of the faecal particles through alternate differential sedimentation and flotation, respectively in water and 40% sucrose solution, ending with a watery suspension (Reinecke 1973). For final 'cleaning' of the ova, the suspension of ova and faecal particles is poured into a flat-bottomed glass crystallising dish to a depth of about 3 cm and left to stand for about a minute before the contents of the dish are carefully poured at a slow and even pace into a second dish of the same sort. The ova, having a higher relative density than most of the faecal particles, sink relatively rapidly to the bottom and many of them adhere to the dish, whilst the majority of the faecal particles remain in the suspension that is poured off. The ova are harvested by washing into a beaker, with the use of a wash bottle. The process is repeated until few ova, appearing to the naked eye as a whitish, granular layer on the bottom of the dish, are visible after decantation.

The cleansed *Nematodirus* spp. ova are next cultured in water containing a pinch of potassium dichromate ($K_2Cr_2O_7$) to control fungal growth until the L_3 have emerged. Continual aeration of the suspension seems to be beneficial for stimulating hatching. For *Nematodirus spathiger*, the yield of L_3 can be improved considerably by storing the ova at about 4 °C for a week before incubation (Viljoen 1972). In the case of *Nematodirus battus*, formalin is added weekly to the culture over the 5–6 weeks of incubation to a final concentration of 1%, incubating the ova for an hour and then washing them on a 53 µm sieve before continued incubation (F. Jackson pers. comm., 1998 – Anonymous, 'Moredun Research Institute Parasitology Division standard operating procedures').

Nematodirus spp. ova can also be 'hatched' artificially (F. Jackson pers. comm., 1998 – Moredun SOPs): well cleansed, fully embryonated ova (i.e. on the point of hatching) are concentrated to obtain about 1 mL of solid material (eggs) per 10 mL of water. About 0.25 mL of egg suspension is pipetted in a line on a thick glass base plate measuring 300 mm × 200 mm × 8 mm, covered with a similar 'crushing' glass plate and firm pressure is applied on the top plate until the ova are felt to crack. Stereoscopic examination should reveal that almost all the eggs have cracked, with larvae emerging. The larvae emerge fully when the preparation is re-suspended in water.

Larva preservation

Most L_3 that die from natural causes or are killed (e.g. with heat) become granular and less translucent in appearance and tend to curl up, such as when killed with formalin. However, if alive, they can be relaxed and preserved practically unchanged in appearance for training and reference purposes. Formalin is added to a final concentration of about 1% – 2% to the suspension of larvae, which are then killed by heating the suspension to 55 °C – 57 °C for about a minute (note that most of the common infective larvae can survive for even some hours in much higher concentrations of formalin than the amount described above). Larvae killed and preserved in this way do not curl up, largely retain their brightly translucent appearance and their internal structures are more clearly discernable than those of L_3 killed by heating alone (Van Wyk *et al*. 2004).

Larva preparation for identification

A drop of larval suspension is deposited on a glass microscope slide and the larvae killed with Lugol's iodine solution that is pre-diluted to a level where it takes a few minutes before the L_3 become darkly stained. The reason for this is that it is more difficult to observe internal structures of larvae (such as the shape of the oesophagus that is important in some cases) and also to differentiate free-living nematodes from L_3 of *Bunostomum* and *Gaigeria* spp. (which rapidly stain almost uniformly brown over their entire length) from the majority of the others, in which the cranial part of the larva initially stains considerably less intensively than the rest.

Larva identification

Note that, in this article, the tip of the cranial extremity of a larva is referred to as its 'head' and the caudal tip as its 'tail' (Figure 1) and the free sheath beyond the tail tip as the STE.

Morphological identification of L_3 of most parasitic nematodes is based principally on examination of the caudal and cranial extremities, although other features such as the length or shape of the oesophagus or cranial refractile spots are important in some genera. Note, however, that once exsheathed, L_3 of relatively few genera can be differentiated. Even when a space has formed between the cranial tip and the sheath of an aging larva, the characteristic shape of the head appears distorted and more squared than usual, thus increasing the chances of incorrect identification.

Length of sheath tail extension

The length of the STE is a very important criterion for identification and, to facilitate its application in larval differentiation, the STE of every larva being evaluated is related to that of *Tr. colubriformis* and *Tr. axei*. To this end, the length of the STE ('c' in Figure 1) of *Tr. colubriformis* and *Tr. axei* (± 33 µm) is represented by 'X', to which that of each L_3 encountered in a culture is related as follows:

Length of STE of L_3 being identified ('c' in Figure 1) = STE / 'X'.

Proportion of sheath tail extension comprising a filament

If the STE ends in a narrow, thin filament ('d' in Figure 1), the proportion of the total length of the STE that this comprises is calculated, although with practice it can usually be estimated without the need for measurement. Note, however, that there is no exactly definable point of transition from the sheath filament to the cranial portion of the STE per genus or species; it is invariably a more or less gradual process, with no precise point of inflexion. On the other hand, because of relatively large differences in filament proportion between those species or genera such as *Chabertia ovina* and *Oesophagostomum* spp. that are difficult to differentiate (Table 1), this fact does not detract from its usefulness as a criterion for identification.

Individual values in Tables 1 and 2 were derived as described by Van Wyk *et al*. (2004). In some cases, the lengths of the STEs were not listed in the papers reviewed by them, but could be calculated from the tables, estimated from photographs and/or figures drawn to scale, or could be calculated from histograms (Keith 1953).

Differential larval count

Identification procedure

Firstly, a stage micrometer is used to determine, for each microscope objective lens, the number of divisions of the graticule in the ocular lens that span 33 µm; that is, the mean length of the STEs of *Tr. axei* and *Tr. colubriformis*, defined as 'X' for the purposes of the present system of identification (Table 1). Then the STEs of the L_3 encountered in the diagnostic samples are compared, in turn, to the number of divisions (usually four divisions at 100× magnification) recorded for *Tr. colubriformis* and *Tr. axei*. Whilst the experienced person will use 10× ocular and objective microscope lenses (i.e. about 100× total magnification) or even somewhat less for the largest part of each differential count, about double this magnification will be necessary for the exceptional larval specimens that are difficult to identify, as well as for routine differentiation by the novice.

Conventionally, the first 100–200 L_3 encountered per count are identified for estimating the proportion of a given sample which each genus comprises. Whilst L_3 can generally be identified only to genus, this is not absolute. Because the L_3 of *Trichostrongylus* spp. of small ruminants are difficult to differentiate from those of *Teladorsagia* spp., they are commonly grouped during the count. However,

TABLE 1: Measurements of third-stage larvae of small ruminants, including sheath tail extension[a], 'X'-values and the proportion of the sheath tail extension comprising a filament.

Nematode	Length of STE (µm)		'X'-value of STE		Filament (% of STE)[b]
	Mean	Range	Mean	Range	
Trichostrongylus spp.[c]	30	18–32	1.0	0.6–1.1	Nil
Trichostrongylus falculatus and *Trichostrongylus rugatus*	51	46–56	1.7	-	Nil
Haemonchus contortus	74	65–82	2.5	2.2–2.7	10–15
Teladorsagia circumcincta	35	30–44	1.2	1.0–1.5	Nil
Cooperia spp. (mainly *Cooperia curticei*)	46	39–52	1.5	1.3–1.7	20–25
Cooperia oncophora	73	62–82	2.4	2.1–2.7	20
Cooperia spp. (from antelope)[d]	58	-	1.9	-	-
Oesophagostomum venulosum	168	122–207	5.6	4.1–6.9	-
Oesophagostomum columbianum	153	125–188	5.1	4.2–6.3	60–70
Chabertia ovina	123	101–150	4.1	3.4–5.0	25
Bunostomum trigonocephalum	99	85–115	3.3	2.8–3.8	40–50
Gaigeria pachyscelis	132	128–135	4.4	4.3–4.5	50
Nematodirus filicollis	261	-	8.7	-	50
Nematodirus spathiger	270	267–309	9.0	8.9–10.3	60
Nematodirus battus	171	-	5.7	-	-

STE, sheath tail extension.
[a], For sources from which the values in the table were obtained, see Van Wyk, J.A., Cabaret, J. & Michael, L.M., 2004, 'Morphological identification of nematodes of small ruminants and cattle simplified', *Veterinary Parasitology* 119, 277–306. http://dx.doi.org/10.1016/j.vetpar.2003.11.012, PMid:15154594
[b], For calculation of mean 'X'-values, the mean of *Trichostrongylus* spp. of sheep was used (Van Wyk *et al*. 2004 – see above).
[c], Proportion of the STE that is filamentous (J. Van Wyk pers. obs., 1990, unless otherwise indicated in text).
[d], *Cooperia fuelleborni*, *Paracooperia serrata*, *Cooperioides antidorca* (Mönnig, 1931).

TABLE 2: Measurements of third-stage larvae of cattle, including sheath tail extension[a], 'X'-values and the proportion of the sheath tail extension comprising a filament.

Nematode	Length of STE (µm)		'X'-value of STE[b]		Filament (% of STE)[c]
	Mean	Range	Mean	Range	
Trichostrongylus axei	32	25–41	1.1	0.8–1.4	0
Haemonchus placei	102	80–119	3.4	2.7–4.0	20
Ostertagia ostertagi	65	45–83	2.2	1.5–2.8	10
Cooperia pectinata / punctata	59	37–78	2.0	1.2–2.6	20
Cooperia oncophora	94	65–116	3.1	2.2–3.9	20
Bunostomum phlebotomum	73	58–96	2.4	1.9–3.2	50
Oesophagostomum radiatum	163	136–185	5.4	4.1–6.9	40–45
Nematodirus helvetianus	250	203–283	8.3	6.8–9.4	50
Nematodirus battus	165	-	5.2	-	-

STE, sheath tail extension.
[a], For sources from which the values in the table were obtained, see Van Wyk, J.A., Cabaret, J. & Michael, L.M., 2004, 'Morphological identification of nematodes of small ruminants and cattle simplified', *Veterinary Parasitology* 119, 277–306. http://dx.doi.org/10.1016/j.vetpar.2003.11.012, PMid:15154594
[b], For calculation of mean 'X'-values, the mean of *Trichostrongylus* spp. of sheep was used (Van Wyk *et al*. 2004 – see above).
[c], Proportion of the STE that is filamentous (J. Van Wyk pers. obs., 1990, unless otherwise indicated in text).

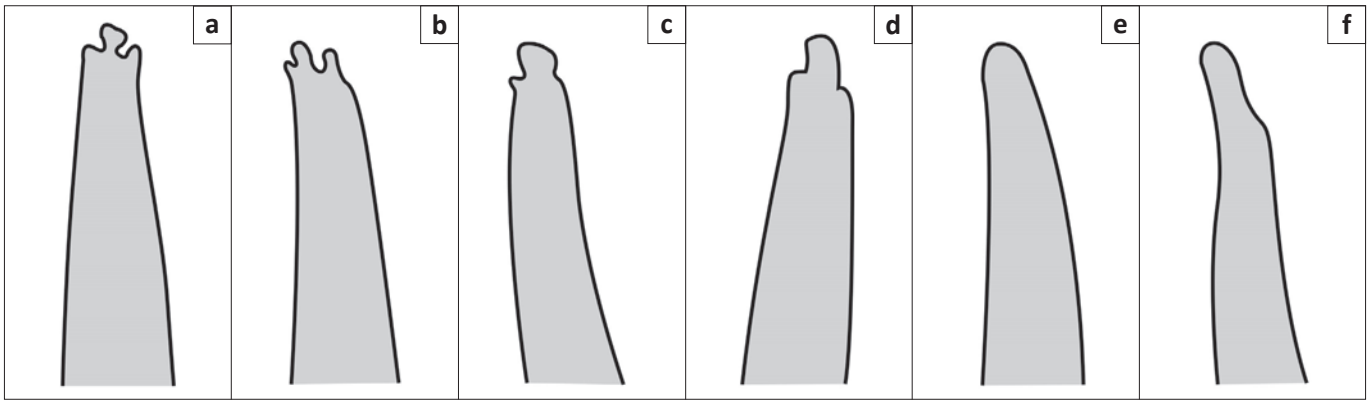
when exsheathed, L_3 of *Nematodirus* spp. and intestinal *Trichostrongylus* spp. can be differentiated from those of *Tr. axei* and *Teladorsagia* spp. in having digitate appendages on the tail, whilst the others have none (Figure 2 [McMurtry *et al*. 2000] and Figure 3).

Whilst exsheathing of intestinal *Trichostrongylus* spp. aids in their identification, larvae that are exsheathed are often difficult to differentiate, as mentioned above. This impasse of having to differentiate some L_3 after they have been exsheathed whilst others must perforce be ensheathed can be overcome to some extent by conducting differential counts in two steps. Initially, ensheathed *Trichostrongylus*, *Teladorsagia* species are grouped, whilst the rest are identified to the level of genus or species. Thereafter, as a second step, the L_3 are exsheathed and only those with specific digitate terminal caudal appendages (Figure 2, intestinal *Trichostrongylus* spp.) are differentiated from the rest of the first 100–200 encountered. Subsequently, the proportion of *Teladorsagia* spp. can be computed as follows:

- Step 1: Do differential count of ensheathed L_3 and let % *Teladorsagia* spp. + *Trichostrongylus* spp. = *x*.
- Step 2: Exsheathe the L_3 and repeat differential count. Let % intestinal *Trichostrongylus* spp. (McMurtry *et al*. 2000) = *y*. Then % *Teladorsagia* spp. + *Tr. axei* = *x* - *y*.

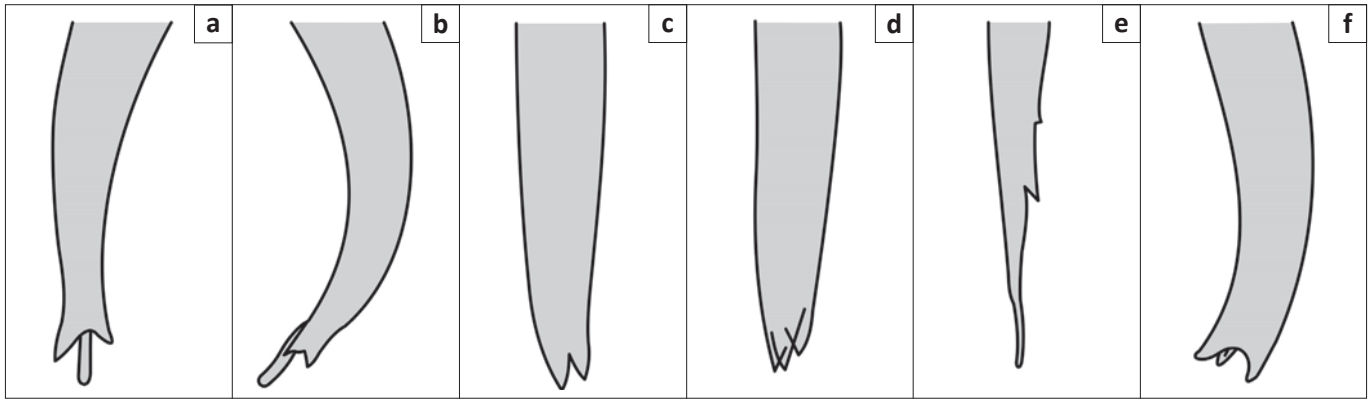
Unfortunately, with the above approach it is not possible to differentiate *Tr. axei* from *Teladorsagia* spp., but if it is important to do so, the method of Lancaster and Hong (1987) can be employed as follows during the first step for a rough estimate, whilst keeping in mind that Cabaret (pers. comm., 2003) experienced the Lancaster and Hong (1987) technique to have a wide margin of error (see discussion below):

- Step 1: Do differential count of ensheathed L_3 and let % *Teladorsagia* spp. (Lancaster & Hong 1987) = *x*; and let % *Trichostrongylus* spp. (Lancaster & Hong 1987) = *y*.
- Step 2: Repeat differential count after having exsheathed the L_3 and let % intestinal *Trichostrongylus* spp. (McMurtry *et al*. 2000) = *z*. Then % *T. axei* = *y* - *z*.



Source: Drawn from McMurtry, L.W., Donaghy, M.J., Vlassoff, A. & Douch, P.G.C., 2000, 'Distinguishing morphological features of the third larval stage of ovine *Trichostrongylus* spp.', *Veterinary Parasitology* 90, 73–81. http://dx.doi.org/10.1016/S0304-4017(00)00230-2

FIGURE 2: Identification to species level of *Trichostrongylus* spp., based on differences in the morphology of the tips of the larva tails: (a, b) *Trichostrongylus colubriformis*, (c, d) *Trichostrongylus vitrinus*, (e) *Trichostrongylus axei* and (f) *Teladorsagia circumcincta*.



Source: Drawn from Van Wyk, J.A., Cabaret, J. & Michael, L.M., 2004, 'Morphological identification of nematodes of small ruminants and cattle simplified', *Veterinary Parasitology* 119, 277–306. http://dx.doi.org/10.1016/j.vetpar.2003.11.012, PMid:15154594

FIGURE 3: Terminal appendages of *Nematodirus* spp. exsheathed third-stage larvae: (a) *Nematodirus spathiger*, lateral view, (b) *Nematodirus spathiger*, dorso-lateral view, (c) *Nematodirus filicollis*, lateral view, (d) *Nematodirus filicollis*, ventro-lateral view, (e) *Nematodirus battus*, lateral view and (f) *Nematodirus abnormalis*, lateral view.

Ethical considerations

This article is based on research conducted in the 1970s and early 1980s. Whilst it was carried out strictly in accordance with ethical considerations overseen by the management of the Onderstepoort Veterinary Research Institute, at that stage there were no national or international guidelines for work of this nature of which the authors were aware. In 2004, a novel method of larvae evaluation was identified and described from the work of Van Wyk *et al.* (2004) and the present study illustrates this in terms of the differences between the larvae, whilst, at the same time, accurately drawing the STE filaments (this having been developed only recently, but without the need for further research in animals).

Results and discussion

Differentiation of larvae

Most importantly, as emphasised by Mönnig (1931), is the selection of only those points of comparison between L_3 of the various genera and species that will enable swift identification with the minimum number of measurements. It is also necessary to acquaint yourself with the variation in the appearance of the various features under different depths of focus of the microscope.

It is exceedingly difficult to photograph larval STEs such that both the tip of the STE and the rest of the caudal extremity of a given larva is in focus simultaneously. Hence the excellent photographs in the papers of Corticelli and Lai (1964) and Henriksen (1972), and to some extent also of Keith (1953), deserve particular mention, as these workers succeeded particularly well, and their photos can be put to very good use when training inexperienced persons to identify L_3. On the other hand, some of the modern electronic photomicroscopes are able to integrate a series of photos at different depths of focus into a composite picture with the entire sheath tail in focus and this could be used fruitfully to this end.

Tables 1 and 2 contain summaries of the mean measurements and corresponding 'X'-values of the different common worm genera and/or species (see Van Wyk *et al.* 2004, Tables 1–7, for details gleaned from the literature). L_3 of parasitic nematode genera are illustrated in this article in Figures 2–13.

As discussed by Van Wyk *et al.* (2004), in most cases where drawings were published previously, the shapes of the heads (cranial extremities) of the larvae and the proportional lengths of the filamentous portions of the STEs were not drawn accurately to scale. Many of the differences between worm genera are very small, making it difficult to observe

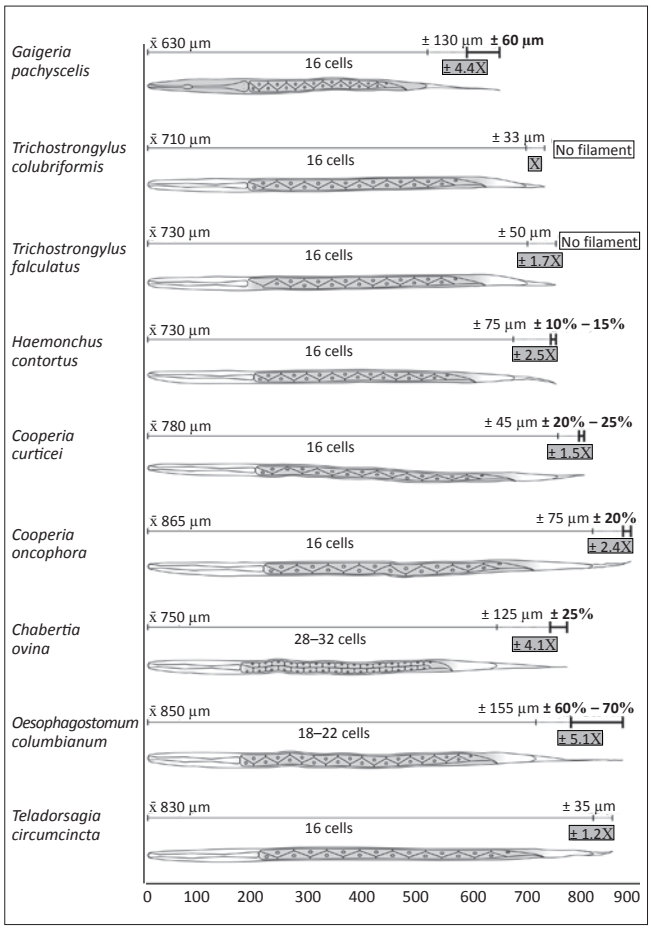


FIGURE 4: Third-stage larvae of common nematodes of small ruminants, measured in micrometres (µm).

and even more difficult to draw accurately. There are notable exceptions, such as the drawings of Borgsteede and Hendriks (1974), which are relatively accurate regarding the morphology of both the heads and STEs of the L_3; yet, identification remained so difficult, especially for the inexperienced, that another morphological feature was sought that could simplify the process. The sheath tail filament presented this possibility.

The potential of the STE filament for differentiation of L_3 was not recognised previously. Most earlier workers either did not take it into consideration as a distinguishing feature, or it was mentioned in general without investigating its potential for identifying L_3 to the level of genus or species (Corticelli & Lai 1964; Gibbons *et al*. 2012). This omission could perhaps be ascribed to the fact that, as mentioned, the transition between the filament and the rest of the STE is not sharp and thus cannot be pinpointed with certainty, especially because it is also affected by the depth of focus of the microscope.

With few exceptions, such as some *Nematodirus* spp. and intestinal *Trichostrongylus* spp., the infective larvae of the common nematodes of domestic ruminants can be identified only to genus level. However, as differences between species of a given genus in characteristics such as pathogenicity and, to some extent, susceptibility to anthelmintics are generally small, this limitation is seldom a serious disadvantage.

Morphological differences between some genera (e.g. *Trichostrongylus* spp. and *Teladorsagia* spp.) are so small that

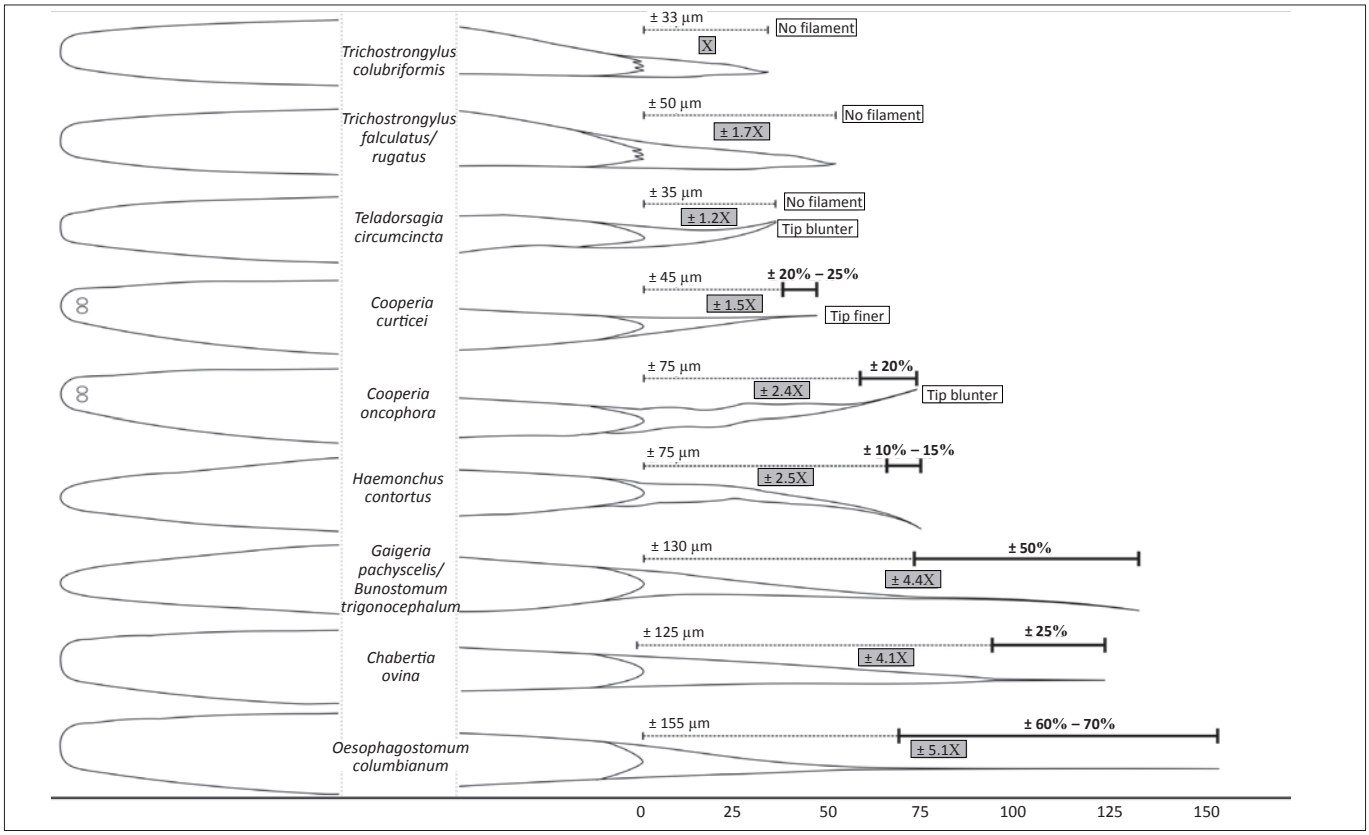


FIGURE 5: Cranial and caudal extremities of third-stage larvae of common nematodes of small ruminants, measured in micrometres (µm).

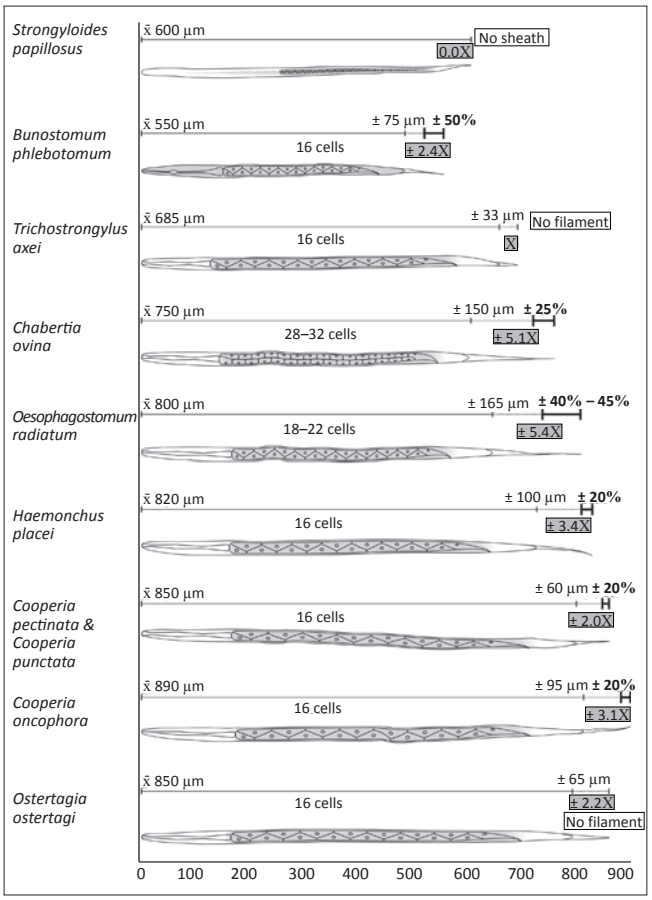


FIGURE 6: Third-stage larvae of common nematodes of cattle, measured in micrometres (µm).

the 'X' system is ineffective for differentiation and the STEs have no filaments. However, these are the exceptions, as the differences are mostly large enough to make it possible after a few measurements (in divisions on the graticule) of L_3 in a mixed culture to judge the various 'X'-values without the necessity for many (if any) further measurements. Experience has shown that even relatively small differences can be recognised without recourse to measurement. For instance, whilst this system was in the early stages of development we became alerted, without having to resort to measurements, to a pure culture of *Trichostrongylus falculatus* (the sheath tail of which differs from the common *Trichostrongylus* spp. by only 0.5 'X') when faecal samples were submitted after a field outbreak of deaths in sheep in the Free State Province. On the other hand, variations within species or genera (Tables 1 and 2) must be kept in mind and it remains essential to take note of and to use other morphological features, such as the shape of the head (Figures 5–8, 14 and 15) and the refractile bodies in the heads of *Cooperia* spp. (below), in addition to the 'X' system. It is also advisable to have L_3 of at least *Tr. colubriformis* and/or *Tr. axei*, but preferably of more genera, in pure culture available in each laboratory where either diagnostic or research differential larval counting is performed. Only small numbers of these L_3 are required at a time, with the result that a single batch killed and preserved as described above can suffice for years.

Figures 14 and 15 are organograms that can function as illustrated, interactive keys for the stepwise morphological

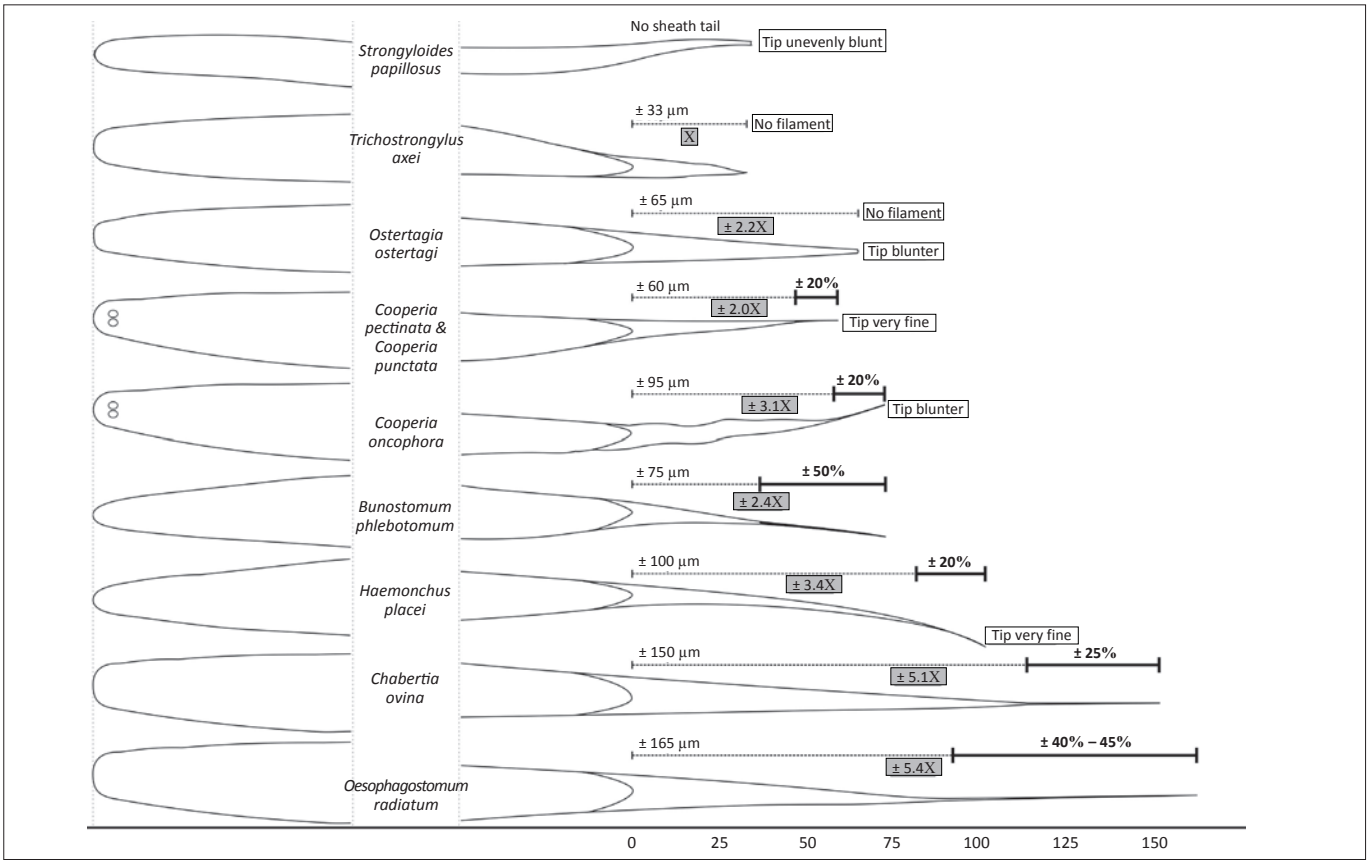


FIGURE 7: Cranial and caudal extremities of third-stage larvae of common nematodes of cattle, measured in micrometres (µm).

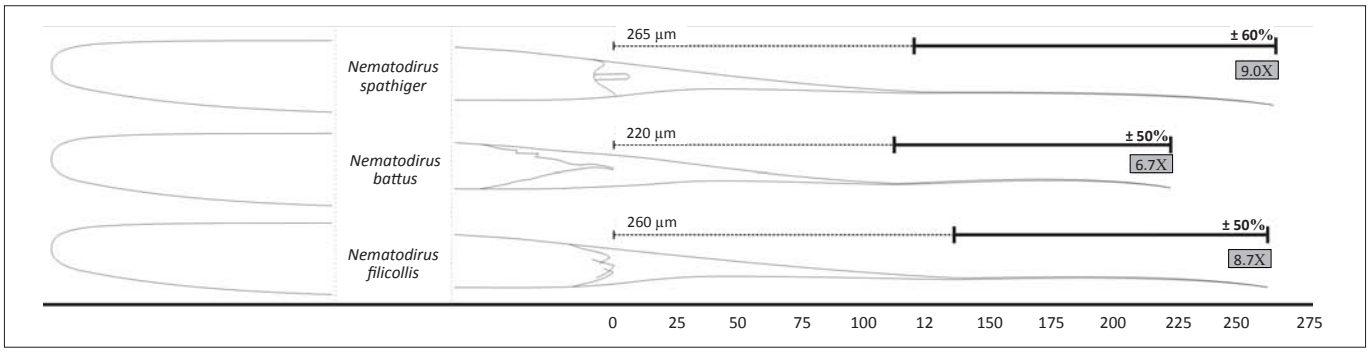
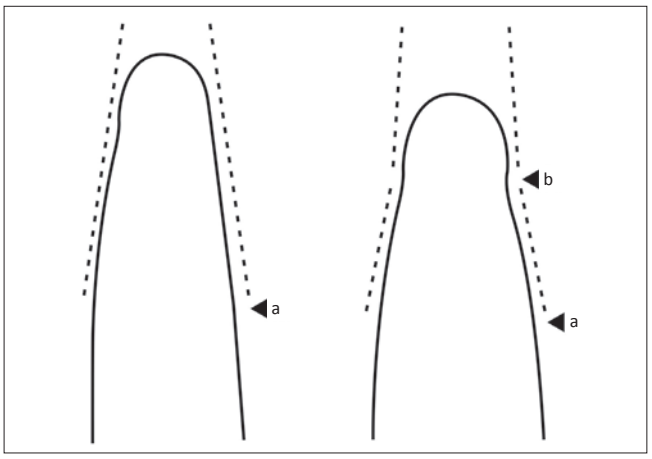


FIGURE 8: Cranial and caudal extremities of ensheathed third-stage larvae of *Nematodirus* spp., measured in micrometres (µm).



Source: Drawn from Lancaster, M.B. & Hong, C., 1987, 'Differentiation of third stage larvae of "ovine *Ostertagia*" type and *Trichostrongylus* species', *The Veterinary Record* 120, 503. http://dx.doi.org/10.1136/vr.120.21.503, PMid:3604011

FIGURE 9: Differentiation of third-stage larvae of *Trichostrongylus* spp. (left) and *Ostertagia* spp. (right), depicting (a) initial point of inflexion craniad and (b) 'shoulder' of *Ostertagia* spp.

Source: Reproduced from Van Wyk, J.A., 1977, 'A rapid method for differentiating between the infective larvae of *Oesophagostomum columbianum* and *Chabertia ovina*', *Onderstepoort Journal of Veterinary Research* 44, 197–199. PMid:614536

FIGURE 10: Exsheathed third-stage larva of *Oesophagostomum columbianum* with a total of 20 or 21 intestinal cells (larva thawed after having been frozen in liquid nitrogen).

identification of common nematode L_3 of small ruminants and cattle, respectively. They were compiled largely for the purpose of training of the inexperienced and are intended for use together with the various figures depicting prominent distinguishing features of the morphology of the L_3 concerned.

Salient features of L_3 of the common nematode genera and/or species

Trichostrongylus spp.

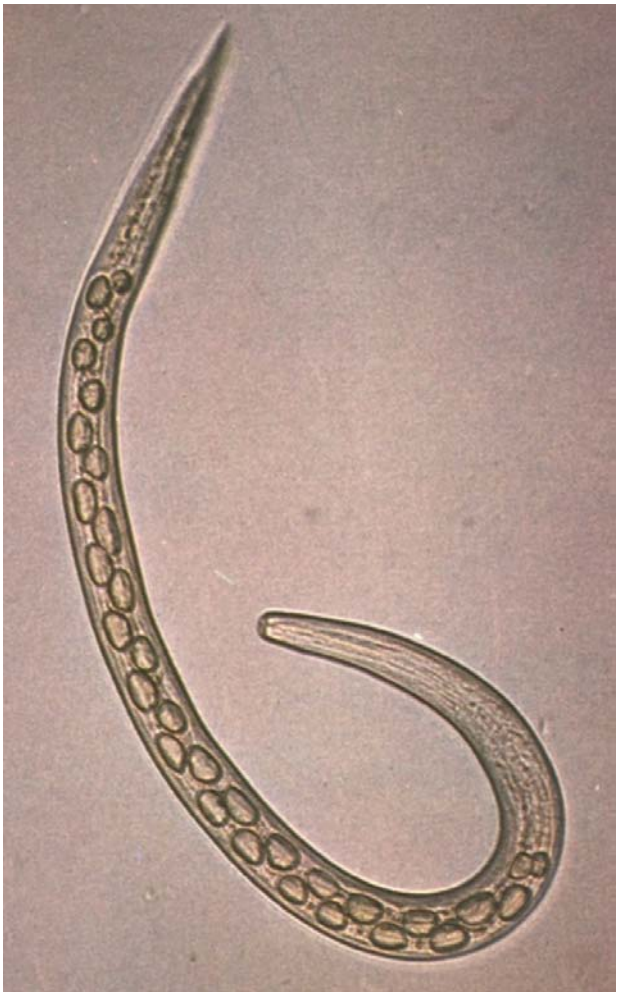
Whilst very similar, there are some distinguishing features between different *Trichostrongylus* species of domestic ruminants, for instance in length of STE (e.g. 'X' and no filament in *Tr. colubriformis* and *Tr. axei* and 1.7 'X' in *Tr. falculatus* and *Trichostrongylus rugatus*) (O'Callaghan 2004; Van Wyk *et al*. 2004) and the presence or absence of digitate appendages on the caudal extremity of the larvae (Figure 2) (McMurtry *et al*. 2000). Common to all the *Trichostrongylus* species included in this article is that the STE is without a filament and tapers so sharply that it resembles the point of a sharpened wooden pencil. The very short STE of *Tr. colubriformis* and *Tr. axei* is used as the basis of the classification system (Van Wyk *et al*. 2004). When exsheathed, the L_3 of *Tr. axei* resemble those of *Teladorsagia* spp. in that the tip of the tail is smooth, in contrast to the irregular protuberances mentioned for intestinal *Trichostrongylus* spp. However, the differences can be discerned only at high magnification and are usually visible only in larvae that are exsheathed. Hence, as mentioned above, this feature requires a second step in the differential larval count procedure.

Ostertagia and *Teladorsagia* spp.

Small ruminants: The STE of the *Teladorsagia* spp. of small ruminants overlaps considerably in length with that of *Trichostrongylus* spp. and is very similar in appearance, resembling a sharpened pencil point, and both are without a terminal filament (Table 1). Added to this is that, as mentioned, the tip of the tail of the *Teladorsagia* spp. L_3 is smooth, similar to that of *Tr. axei*. According to Lancaster and Hong (1987), the head of ovine *Teladorsagia* species has a slight 'shoulder' close to its cranial tip (illustrated in Figure 9), whilst that of *Trichostrongylus* spp. does not. This is fully described and depicted in Lancaster and Hong (1987) and Van Wyk *et al*. (2004). Note, however, that J. Cabaret

(pers. comm., 2003) reported an average of 30% incorrect identifications with use of this method for differentiating *Tr. colubriformis* and *Teladorsagia circumcincta* in mixed culture.

Whilst *Tr. axei* and *Te. circumcincta* do differ markedly in total length (with respective means of 720 µm and 820 µm), it is time-consuming to do such measurements. Furthermore, the lengths of the L_3 of *Trichostrongylus* spp. and *Teladorsagia* spp. overlap to a considerable extent (O'Callaghan 2004), thus making it a relatively impractical for accurate discrimination.



Source: Reproduced from Van Wyk, J.A., 1977, 'A rapid method for differentiating between the infective larvae of *Oesophagostomum columbianum* and *Chabertia ovina*', *Onderstepoort Journal of Veterinary Research* 44, 197–199. PMid:614536

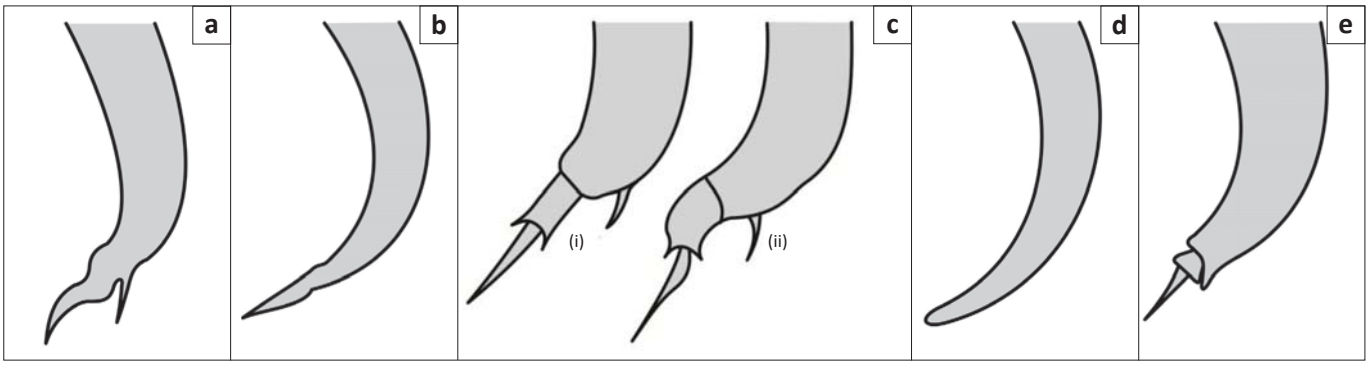
FIGURE 11: Exsheathed third-stage larva of *Chabertia ovina*, with a total of 32 intestinal cells (larva thawed after having been frozen in liquid nitrogen).

Cattle: In this host species, in contrast to the situation in small ruminants, there is small chance of confusing the STEs of *Ostertagia ostertagi* and *Trichostrongylus* spp. L_3. At slightly more than 2 'X', the STE of L_3 of *Os. ostertagi* is twice as long as that of the common *Trichostrongylus* spp. and the presence of a short filament serves further to differentiate them from the L_3 of both *Te. circumcincta* and *Trichostrongylus* spp. (Tables 1 and 2). Another useful distinguishing feature is that the STE of *Os. ostertagi* ends in a blunter tip than that of *Haemonchus placei* (Borgsteede & Hendriks 1974) (Figures 6 and 7).

Haemonchus spp.

As discussed by Van Wyk *et al*. (2004), there has been confusion in the past concerning the validity of *H. placei* as a separate species from *Haemonchus contortus*, despite clear morphological and/or biological distinguishing features between the two both in the L_3 and adult worms. The confusion was compounded by cross-infectivity of both species to sheep and cattle. The adults of the two species are indeed relatively difficult to differentiate morphologically, but with use of the novel approach to differentiation presented now, this should not apply to the L_3.

Whilst the infective larva of *H. contortus* of small ruminants has an STE with a length of 2.2–2.7 'X' and a filament comprising 10% – 15% thereof, the corresponding values of *H. placei* of cattle are 2.7–4.0 'X' and ± 20% (Figures 4–7 and Tables 1 and 2). On the other hand, Borgsteede and Hendriks (1974) discuss the possibility of confusion between the L_3 of *H. placei* and of *Os. ostertagi* in cattle and point out the respective bullet-shaped and flatter heads and much finer and blunter STE tips of *Os. ostertagi* and *H. placei* (see Figures 6 and 7 in this article, as well as subsection 3.1.3. of Van Wyk *et al*. 2004 and, particularly, Figure 3 of Borgsteede & Hendriks 1974). Added to this is that the filament comprises a larger proportion of the STE of *H. placei* than it does in *Os. ostertagi* (Table 2) (Van Wyk *et al*. 2004) and, as discussed



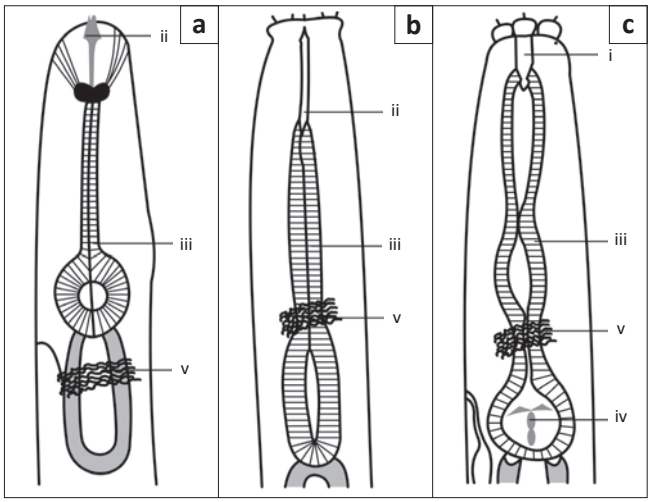
Source: Drawn from Van Wyk, J.A., Cabaret, J. & Michael, L.M., 2004, 'Morphological identification of nematodes of small ruminants and cattle simplified', *Veterinary Parasitology* 119, 277–306. http://dx.doi.org/10.1016/j.vetpar.2003.11.012, PMid:15154594

FIGURE 12: Tail morphology of lungworm larvae of five genera: (a) *Muellerius capillaris*, (b) *Protostrongylus rufescens*, (c) *Cystocaulus ocreatus*, (d) *Dictyocaulus filaria* and (e) *Neostrongylus linearis*, with (i) and (ii) illustrating the different types of appendices to the larva tails.

by Van Wyk *et al*. (2004), the intestine of *Haemonchus* spp. is reported to end caudally in two terminal cells, compared to only one in *Ostertagia* spp.

Cooperia spp.

Most striking is the presence of two unique refractile bodies in the head of the L_3 of *Cooperia* spp. (Figures 4–7), constituting an important distinguishing feature from those of other genera. On the other hand, the STEs of *Cooperia pectinata* and *Cooperia punctata* of cattle somewhat resemble those of *Os. ostertagi* and *H. placei*, all having a filament of about 20% (Table 2). However, in addition to the refractile bodies of *Cooperia* spp., the head of the L_3 of *Os. ostertagi* is considerably more squared than that of *Cooperia* spp. or *H. placei*. In contrast, the STE of *Cooperia oncophora* in cattle is ± 3 'X', more closely resembling the L_3 of *H. placei* than that of *Os. ostertagi*. Another distinguishing feature of *C. oncophora* is that the caudal tip of the sheath of *C. oncophora* is clearly perceptible, whereas that of *C. punctata* 'appears to vanish into nothingness' (Borgsteede & Hendriks 1974), or is 'refractile in appearance' (Anonymous 1977). Borgsteede and Hendriks (1974) also describe the head of the L_3 of *C. oncophora* to be somewhat broader than that of *C. punctata*, although we have found this difference to be difficult to visualise.



Source: Drawn from Van Wyk, J.A., Cabaret, J. & Michael, L.M., 2004, 'Morphological identification of nematodes of small ruminants and cattle simplified', *Veterinary Parasitology* 119, 277–306. http://dx.doi.org/10.1016/j.vetpar.2003.11.012, PMid:15154594

FIGURE 13: Morphology of the cranial extremities (heads) of free-living nematodes: (a) Tylenchida, (b) Dorylaimida and (c) Rhabditida, depicting, (i) stoma, (ii) stylet, (iii) oesophagus, (iv) valve and (v) nerve ring.

Notably, as reviewed by Van Wyk *et al*. (2004), the mean lengths of the STEs of *C. oncophora* L_3 cultured from sheep differ significantly from those from cattle. The respective lengths are about 73 µm (2.4 'X', Table 1) and 94 µm (3.1 'X', Table 2), with corresponding ranges of 62 µm – 82 µm and 65 µm – 116 µm, hence with little overlapping in the lengths of their STEs, as confirmed by the fact that Hansen and Shivnani (1956) recorded only one measurement of less than 79 µm for *C. oncophora* from cattle and Dikmans and Andrews (1933) recorded a maximum of 82 µm for this species from sheep.

The *Cooperia curticei* L_3 of sheep has an STE more closely resembling that of *C. pectinata* and *C. punctata* than that of

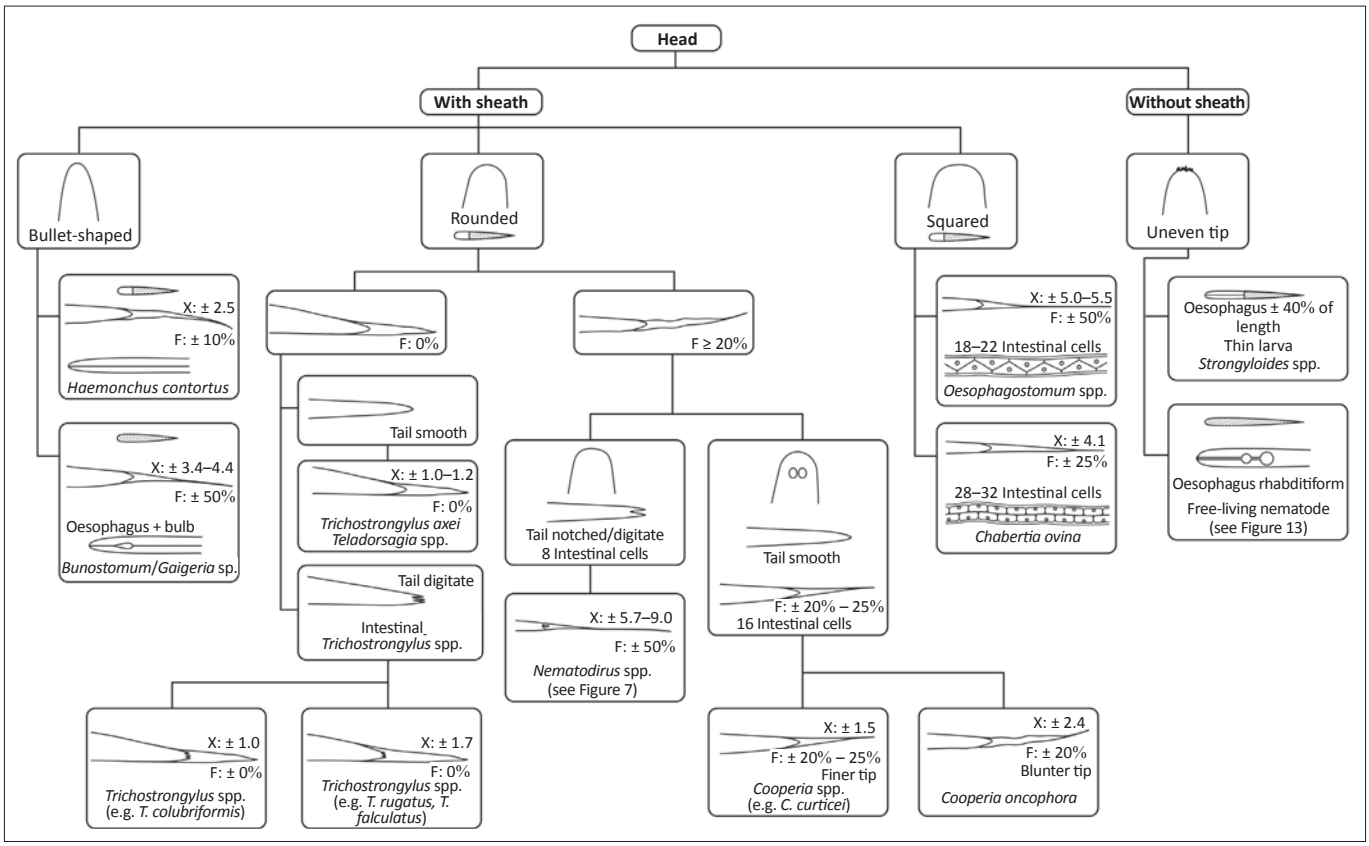


FIGURE 14: Organogram for identifying gastrointestinal nematode and lungworm larvae of small ruminants, including a general description of free-living nematodes.

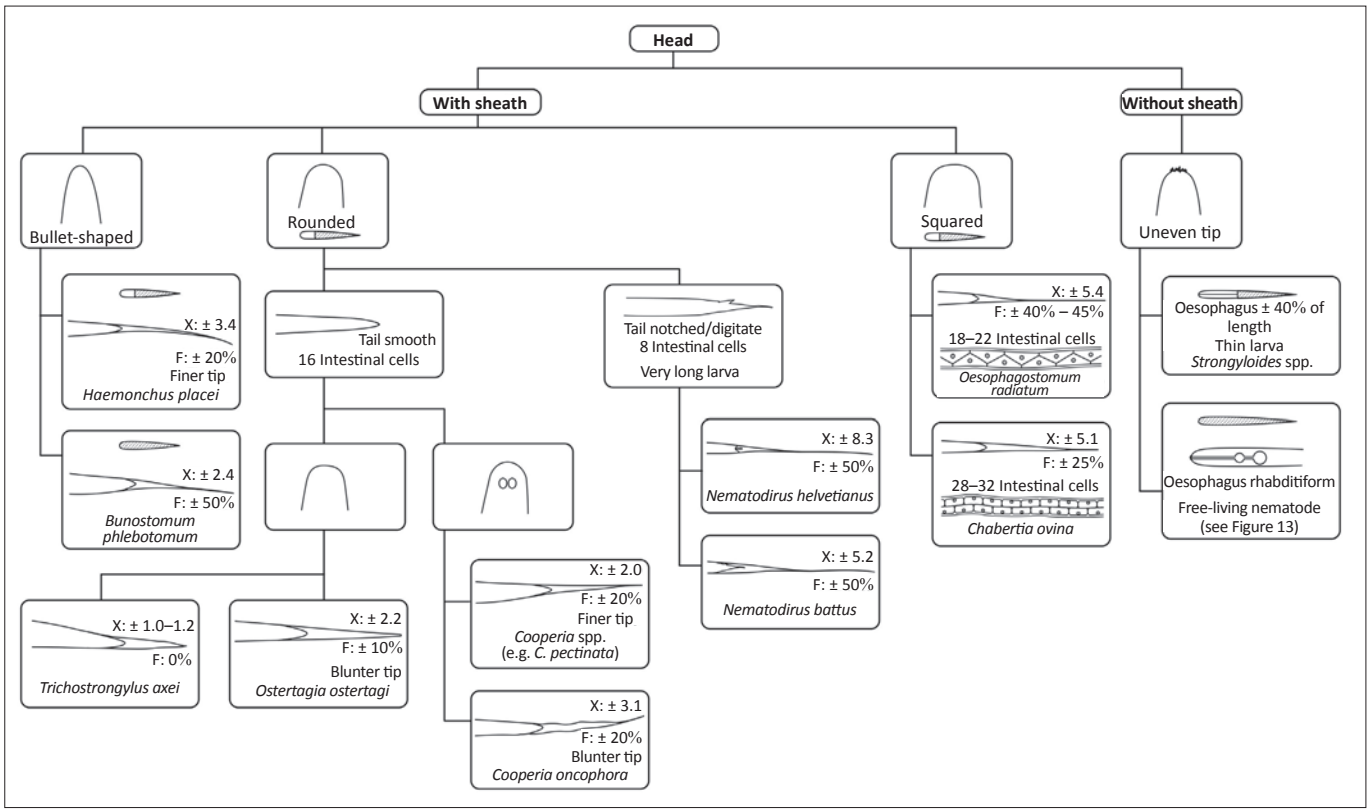


FIGURE 15: Organogram for identifying gastrointestinal nematode and lungworm larvae of cattle.

C. oncophora, but it is important to note that, as mentioned, the STE of the latter is reported to be shorter in small ruminants than in cattle (see above).

The hookworms, *Bunostomum* spp. and *Gaigeria pachyscelis*

Hookworm L_3 (*B. phlebotomum* in cattle, and *Bunostomum trigonocephalum* and *G. pachyscelis* in sheep and goats) are characteristically small in size, being almost 100 µm less in total length than those of *H. contortus*, the second shortest of the common species or genera. They stain uniformly dark with iodine, in contrast to the L_3 of other nematodes that stain light brown cranially and dark brown caudally shortly after addition of the iodine. The STEs vary from 2.4 'X' for *B. phlebotomum* L_3 to 4.4 'X' for *G. pachyscelis*, each with a filament comprising about 50% of the STE. The oesophagus has a prominent bulb caudally that is most easily observed in the live, active L_3, but difficult to visualise after any but very short periods of staining with dilute iodine solution.

The L_3 of the following three genera are set apart from the rest by having conspicuously long STEs.

Oesophagostomum spp. and *Chabertia ovina*

Helminths of these two genera occur widely disseminated in the world, but *Ch. ovina* occurs much less commonly than the other. Whilst in small ruminants *Oesophagostomum venulosum* occurs in Mediterranean-type climate and *Oesophagostomum columbianum* under warmer subtropical and tropical conditions, in cattle *Oesophagostomum radiatum* is practically universally disseminated.

A characteristic of the infective larvae of both genera is that they have relatively long STEs (Table 1 and 2). However, note that the filament of L_3 of *Oesophagostomum* spp. has been observed occasionally to break off, resulting in a much different appearance (Mönnig 1931; J. Van Wyk pers. obs., 1996). Whilst apparently not as yet reported for other species, presumably this is also a possibility and should be kept in mind for both *Ch. ovina* and some others, such as L_3 of *Nematodirus* spp. with long STE filaments. The L_3 of *Oesophagostomum* and *Chabertia* spp. resemble one another so closely that they are described as practically indistinguishable by some authors. However, much of the confusion appears to have resulted from misidentification in the first place, because the numbers and appearance of the intestinal cells, as well as distinguishing features of the STEs present ready methods of differentiation.

Number of intestinal cells: Whilst, in some instances, both *Oesophagostomum* and *Chabertia* genera are described and/or depicted as having 32 rectangular intestinal cells or as being indistinguishable (Anonymous 1977; Eckert 1960; Gibbons *et al*. 2012), in others where only *Oesophagostomum* spp. are described, they are listed as having or perhaps having up to 32 cells (Mönnig 1931). In contrast, Dikmans and Andrews (1933) and Borgsteede and Hendriks (1974) correctly describe *Ch. ovina* to have about 32 and *Oesophagostomum* spp. about 20 intestinal cells, although the latter authors were uncertain whether some L_3 of *Oesophagostomum* could have up to 32 cells.

Unfortunately it is usually only in the very newly developed infective larvae that the shapes of the intestinal cells are

clearly discernable, but even if not, it is sometimes possible to count the cells by examining the granular cell content under relatively high magnification. In addition, a further method has been found to illustrate the number of cells per genus very effectively; when exsheathed L_3 of *Oe. columbianum* and *Ch. ovina* are frozen in liquid nitrogen and subsequently thawed, a bubble can generally be seen in each intestinal cell, very effectively differentiating the two genera (Figures 10 and 11, re-photographed from Van Wyk 1977). The photographs clearly illustrate the difference in numbers of cells, being 18–22 in *Oe. columbianum* and 28–32 in *Ch. ovina*.

Intestinal cell shape: The intestinal cells of the L_3 of the two genera under discussion also differ dramatically in shape, being triangular in *Oesophagostomum* spp. and rectangular brick-shaped in *Ch. ovina* (Figures 4, 10 and 11). However, as discussed, the outlines and thus shapes of the cells are frequently not discernable in any but newly developed larvae.

Sheath tail extension filament: It is in the STE that the most consistent means of differentiation of *Oe. columbianum* and *Ch. ovina* L_3 lies, not in its length, but mainly in the proportion of the length comprising the filament, being about 50% – 60% in the former and 25% in the latter (Table 1). Hence, despite it being difficult to pinpoint the exact point of inflexion between the filament and the cranial, non-filamentous portion of the STE, the margin of error resulting therefrom is small enough to allow accurate discrimination.

In summary, *Oesophagostomum* spp. and *Ch. ovina* L_3 respectively have ± 18–22 and 28–32 triangular intestinal cells, the lengths of the STE are ± 5 'X' and 4 'X' and the filaments constitute from 40% to 70% of the STE by species in the former and 25% in the latter (Figures 5 and 7; Tables 1 and 2).

Nematodirus spp.

The commonly encountered species are *N. spathiger*, *Nematodirus filicollis* and *Nematodirus abnormalis* of small ruminants, *Nematodirus helvetianus* of cattle and *N. battus* of lambs and also calves.

When sheathed, *Nematodirus* spp. L_3 are conspicuously longer than those of other nematode genera. They have only eight large intestinal cells, a considerably longer STE (except for *N. battus*) and prominent digital appendages on the tails of the larvae (Figure 3). It is seldom necessary to identify L_3 of *Nematodirus* spp. in routine faecal cultures because the ova of the genus require at least two weeks to hatch, whilst faecal cultures are usually harvested after 5–7 days and require pre-exposure to low temperatures to be able to hatch (Viljoen 1972). However, because *Nematodirus* ova are much larger than, and hence easily distinguished from, those of most other parasitic nematodes, they can be recorded separately in routine faecal egg counts and any *Nematodirus* L_3 which may be encountered in cultures, are ignored and not included in the differential larval counts. It is important to note, however, that *Nematodirus* ova can be confused in general appearance with those of *M. marshalli*, the ova of which are similarly considerably larger than those of most of the parasitic gastrointestinal nematodes (Soulsby 1982). Also, whilst the eggs of the other common *Nematodirus* species are more or less oval in shape, that of *N. battus* is considerably smaller and much more oblong, resembling 'oversize' eggs of the other common gastrointestinal strongyles.

Strongyloides papillosus

The L_3 of this species are exceptionally thin, the oesophagus comprises about 40% of the total length, the tip of the tail is bifid and it has no covering second sheath as do the L_3 of the other genera, hence also no STE. At the lower magnifications usually used for routine larva identification, it is not possible to see that the tip of the tail is bifid, but it does have the appearance of an STE, of which the tip of the filament has broken off. Misidentification may occur if only the tail of this larva is examined instead of the entire larva, because the disproportionately long oesophagus will be missed and it sometimes seems – mistakenly so – as if it does have an STE.

Dictyocaulus spp.

Both *Dictyocaulus filaria* of small ruminants and *Dictyocaulus viviparus* of cattle are ovo-viviparous. Hence, their ova hatch in their respective hosts and the L_1 are passed in the faeces. For diagnosis, the L_1 are recovered by Baermannisation, otherwise a few faecal pellets or a blob of faeces can be placed in water in a Petri dish and the surrounding water observed under a stereo microscope for larvae migrating out of the faeces.

Dictyocaulus spp. larvae are small in size and very lethargic, the tail ends caudally in a smoothly rounded tip ('d' in Figure 12), no STE is discernable and the intestinal cells are indistinct and usually brown in colour. A striking difference between L_1 of *D. filaria* and *D. viviparus* is a conspicuous bulbous thickening of the sheath on the head of the former that is absent from *D. viviparus*. Similar to the infective larvae of *Nematodirus* spp., larvae of *Dictyocaulus* spp. are seldom encountered in representative numbers in routine larval cultures, but for a different reason; they migrate poorly out of such cultures unless collected as described for the recovery of hookworm larvae. Striking photos of *D. filaria* and *D. viviparus* larvae can be seen on the website of Gibbons *et al*. (2012).

Protostrongylid lungworm larvae

Muellerius, *Cystocaulus*, *Neostrongylus* and *Protostrongylus* spp. are commonly present in sheep faeces in Europe (Cabaret 1986), whilst *Varestrongylus pneumonicus*, which is found in small ruminants in Asia, is recorded only in roe deer in temperate Europe. The L_1 of the protostrongylids are recovered from faeces using a Baermann-derived technique (Cabaret, Dakkak & Bahaida 1980; also various textbooks, e.g. Hansen & Perry 1994). The morphology of the tails of some of the larvae is shown in Figure 12, but persons who

encounter these L_1 more commonly should study the more detailed descriptions in Van Wyk *et al*. (2004) and Gibbons *et al*. (2012).

Free-living nematodes

Cultures of faeces picked up from the ground are often contaminated with free-living nematodes, which then tend to dominate faecal cultures to the extent that it is almost impossible to do a differential parasitic nematode larval count. Thus it is preferable to collect faeces from the rectum for cultures, or from bags hung on the animal, although contamination has also been described despite the use of faecal collecting bags (Van Wyk *et al*. 2004), in which case it was overcome by shearing and thoroughly washing the buttocks of the animals.

It is important to note that, even though they often resemble the L_3 of parasitic nematodes, most of the free-living nematodes encountered in faecal cultures are adults and not larvae. In contrast with the majority of the parasitic larvae, the commonly encountered free-living nematodes stain uniformly, extremely dark brown with iodine, are relatively thick and cigar-shaped, have a rhabditiform oesophagus (i.e. with two conspicuous bulbs caudally) and a long tail with no covering sheath. However, the genera differ markedly from one another (as briefly indicated by Van Wyk *et al*. 2004) and these nematodes differ to such an extent from the parasitic larvae that there should seldom be confusion.

Conclusion

There are dwindling numbers of persons trained in 'conventional' helminthological techniques, such as larva identification, which is relatively difficult to learn even when persons with the required skill are available as mentors to the inexperienced. In addition, whilst the full range of pure cultures (and thus larvae) of the common gastrointestinal nematodes of both sheep and cattle were generally available previously, few laboratories maintain these any longer. Thus, it is essential that every possible aid, such as the newly described use of the proportion of the STE in identification, be available to those who need to learn to differentiate infective larvae obtained by faecal culture.

Acknowledgements

This work was supported by the EU 'PARASOL' (EU-FP6) and 'RISC-NET' projects, the latter under the 'CIDLID' call for applications, funded by a partnership between the Biotechnology and Biological Sciences Research Council, the Department for International Development and the Scottish Government.

Hennie Gerber (now deceased) maintained pure nematode cultures and Lynne Michael and Regina Alves were instrumental in preparations. Jacques Cabaret was the source of information on lungworm larvae, which are described in more detail in Van Wyk *et al*. (2004). Ian Carmichael went to much trouble to locate original material from trials of Beveridge, Martin and Pullman (1985). Michael O'Callaghan drew the 'f' diagram in Figure 3. Hervé Mauleon helped with descriptions of free-living nematodes. Ron Kaminsky, Frank Jackson, Jacques Cabaret, Jacques Cortet, Eric Morgan and Flip van Schalkwyk supplied L_3 of a variety of species.

Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

Authors' contributions

J.A.v.W. (University of Pretoria) was responsible for developing the novel approach to morphological identification of infective larvae of the common gastrointestinal nematode genera of small ruminants and cattle and wrote the manuscript. E.M. (University of Pretoria) was responsible for all the art work and finishing of the article.

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