

Nematode Parasites of Small Ruminants, Camelids and Cattle
Diagnosis with Emphasis on Anthelmintic Efficacy and Resistance Testing

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Part 1. Diagnostic Overview

Summary

Nematode infections of ruminants in Australia and New Zealand usually consist of mixed infections with members of the Trichostrongylidae and related families. Common genera with direct life cycles (that is, without intermediate hosts or vectors) include Haemonchus, Ostertagia, Teladorsagia, Trichostrongylus, Oesophagostomum, Dictyocaulus, and Trichuris. These infections cause losses in production due to morbidity and in some cases mortality. These genera are distributed in both temperate and tropical areas with some minor differences in species compositions in different regions, although they tend to be absent or restricted in arid zones, because the infective stages are susceptible to heat and desiccation.

Identification of nematode infections: *In vivo identification of infections relies on the microscopic detection of nematode eggs in host faeces. Due to close similarities in morphology*

of the eggs of most nematodes, it is necessary to culture faecal eggs to infective third stage larvae for differentiation into genera. Histochemical methods for identification of worm egg species are under development. Post-mortem identification relies on distribution in the organs of the host and morphological features of adult worms (although larval stages can also be identified at least to genus level).

Quantitative methods: *Faecal nematode egg counts (FECs) are used to assess the degree of infection, although there is generally no direct correlation between number of faecal eggs and numbers of adult worms. Traditional manual methods are still standard; attempts to develop semi-automated methods have met with limited success.*

Testing for resistance to anthelmintics: *The resistance by nematodes to all the currently available anthelmintic groups has become widespread and increasingly severe in recent years, especially in sheep, but increasingly in cattle (at least in New Zealand). The egg hatch test (EHT) to detect resistance is still advocated in UK/Europe but is no longer used in Australasia as it is limited to detecting benzimidazole (BZ) resistance, which is nearly ubiquitous in this region. The faecal egg count reduction test (FECRT) is the most widely used on-farm test where faecal egg counts are used to assess the effectiveness of a number of anthelmintic groups (different chemical families) or combinations. The larval development or motility assays (LDA or LMA) are laboratory tests with limited use in detecting macrocyclic lactone (ML) resistance, and commercial kits, which have been marketed in Australia for the past ten years, are no longer generally available. Molecular methods using polymerase chain reaction (PCR) for monitoring resistance to BZ and ML are still limited to research laboratories and are not in routine diagnostic use. Controlled infection and slaughter tests are used for confirmation of resistance and for anthelmintic registration claim purposes.*

Serological tests: *Serological tests are not used for diagnostic purposes because of the frequent occurrence of mixed nematode infections and the cross-reactions that these cause. Faecal antigen detection tests are useful in closely controlled pen infections but are not successful for field use. A commercial ELISA for herd health monitoring of *Ostertagia* infections in cattle has recently been produced in Europe.*

Aetiology

Nematode parasites of small ruminants, South American camelids, and cattle in Australia and New Zealand, are primarily parasites of the gastrointestinal tract, (frequently referred to as gastrointestinal nematodes, GIN), and lungs. A range of nematodes in the families Trichostrongylidae and Dictyocaulidae are usually present as mixed infections. The most important species are those found in the abomasum and small intestine, with few species living in the large intestine or lungs.

Genera include *Haemonchus*, *Cooperia*, *Ostertagia*, *Teladorsagia*, *Bunostomum*, *Trichostrongylus*, *Dictyocaulus*, *Oesophagostomum*, *Chabertia*, *Nematodirus*, *Muellerius* and *Protostrongylus*, the whipworm *Trichuris* (family Trichuridae), and threadworm *Strongyloides papillosus*.

At a species level most are confined to one host only and are not transmissible to other ruminants. However, noticeable exceptions are *Trichostrongylus axei*, which is found in both cattle and sheep (and horses). *Haemonchus contortus*, although a primary pathogen in sheep, can be transmitted to cattle but the more usual *Haemonchus* sp in cattle is *Haemonchus placei* although the latter species is absent from New Zealand.

South American camelids (alpaca and llama) can be infected with GIN from both cattle and sheep, and have a number of unique pathogenic species, such as *Lamanema* in their home countries (Peru, Bolivia, Chile, Argentina etc). These species have not been detected in Australia. A recent report, which is based on morphology of eggs, third stage larvae retained within egg shells and unusual embryonic development, identified what appears to be *Lamanema chavezii* in llamas and alpacas from a Canterbury property in New Zealand.¹ Confirmation must await description of adult worms.

Clinical signs

Most of the clinical signs associated with GIN infections are not specific and can be described as parasitic gastroenteritis (PGE). They are related to intestinal disturbances such as diarrhoea (typically associated with the 'scour' worms *Trichostrongylus* and *Teladorsagia circumcincta* (formerly *Ostertagia circumcincta*)), constipation (*Haemonchus*), and evident as hunched back (*Oesophagostomum columbianum*, nodule worm), poor growth, failure to thrive, rough coat, weight loss, emaciation, anorexia and death.

Clinical signs associated with *Teladorsagia* in small ruminants and with adult worms of *Ostertagia* in cattle (type 1 ostertagiosis) include inappetence, profuse watery diarrhoea (scours) and rapid weight loss. Heavy infections, particularly if accompanied by *Trichostrongylus* spp in sheep and goats, can cause profuse scouring, illthrift and possibly deaths.

The blood-sucking species *Haemonchus* and *Bunostomum* can result in anaemia, elevated temperature, melena, submandibular oedema ('bottle jaw') and even sudden death. Other clinical signs of *Bunostomum* sp in cattle include inappetence, illthrift and prostration, while infection in calves maintained in wet, muddy conditions can be associated with skin penetration by the infective larvae, resulting in feet stamping and licking the legs.

Clinical signs associated with *H. contortus* include anaemia and hypoproteinaemia (seen as submandibular oedema). In South Africa, the Famacha[®] system of standard colour charts is used for assessing/scoring the level of anaemia by comparison of the colour of the inner lower eyelid and is used for tactical treatment of heavily infected sheep. Attempts are being made to assess the usefulness of this system in Australia in *Haemonchus*-endemic regions of northern NSW. In heavy and rapid infections, even animals in fat condition may die relatively quickly. Scouring is

not a feature in sheep and goats unless the parasite infection is mixed and includes 'scour worms' (notably *Teladorsagia* and *Trichostrongylus* spp).

Usually it is the young weaners of all hosts that are most susceptible to GINs; older stock, with the exception of periparturient and lactating ewes, are relatively more resistant. Breeds of sheep differ in their susceptibility to nematodes; some exotic breeds, such as Javanese thin tailed, Florida, and Red Masai, show some resistance while the Merino is highly susceptible to *Haemonchus*. Little natural immunity develops until sheep are 9-12 months old. Weaner calves are the main group of cattle at risk, but provided they experience a gradual exposure to infective larvae from pasture most cattle develop a sufficient level of resistance by the time they are 18 months of age.

Trichostrongylus colubriformis and *T. vitrinus* commonly occur in mixed infections with *Teladorsagia circumcincta*, producing similar clinical signs (inappetence, weight loss and scouring). Intake of *Trichostrongylus* larvae is believed to be the primary agent responsible for 'hypersensitivity scouring' in sheep in the winter-dominant rainfall areas of Victoria/South Australia and south-western Western Australia.

Cooperia spp are widespread but relatively uncommon and non-pathogenic parasites in sheep. *Cooperia* are generally not considered to be particularly pathogenic in cattle but very heavy infections of *C. oncophora* in young weaners may cause a severe gastritis. Clinical signs associated with *Cooperia* spp are typical of those of parasitic gastroenteritis (PGE), and include inappetence, intermittent and watery diarrhoea, and weight loss.

Nematodirus spathiger is a very common parasite of young Australian sheep, and usually relatively non-pathogenic, unlike the situation in New Zealand where this parasite inexplicably has become more important since the 1960s. Heavy infections with severe blackish-green to yellow diarrhoea and illthrift with mortalities can be seen in young sheep.

Clinical signs associated with lungworm infections (*Dictyocaulus* and *Muellerius*) are not pronounced in Australasia, unlike in Europe where the characteristic 'husk', frequent coughing, dyspnoea and parasitic bronchitis can often result in a severe disease in calves.

Signs associated with heavy *Strongyloides* infections are usually restricted to young calves in unhygienic conditions. These signs are anorexia, weight loss and slight to moderate anaemia. There may be respiratory distress (dyspnoea) caused by larvae migrating through the lungs, lassitude, and lameness. *S. papillosus* is commonly found in young dairy calves. Clinical signs include dull demeanour, inappetence, harsh cough and diarrhoea.

O. columbianum (nodule worm) and *O. venulosum* (large bowel worm) occur in sheep and goats. Meat processors can suffer significant financial losses due to condemnation of intestines ('runners') affected by *Oesophagostomum*-associated 'pimply gut'. Clinical signs in heavy infections include variable diarrhoea, emaciation, a humped appearance and stiff gait. Intussusception has also been reported.

Heavy infections of *O. radiatum* in cattle may cause severe clinical disease in young animals with signs including inappetence, illthrift, intermittent diarrhoea, anaemia, emaciation and death.

Clinical signs in sheep severely affected with *Chabertia ovina* include faeces that are soft, often mucoid and perhaps blood-flecked. Illthrift may occur.

Trichuris spp are considered harmless except in very heavy infections.

Epidemiology

All the major GIN and lungworms of ruminants are transmitted by direct life cycles, by the ingestion of infective third stage larvae (L3) from pasture. The whipworms *Trichuris ovis* and *Trichuris globulosa* are slightly different in that they are transmitted by ingestion of the embryonated thick-shelled egg. *Strongyloides papillosus*, *Bunostomum trigonocephalum* and *Bunostomum phlebotomum* can also infect by skin penetration. While the life cycles are direct and generally have a period of about 3 weeks from ingestion of infective stage to mature adult egg-producing worms (prepatent period, PPP), some species notably *H contortus* and *H placei* can have a shorter 14-16 days PPP, and *Strongyloides* sp 10-12 days. The PPP for *Bunostomum* sp is significantly longer at 6 to 10 weeks depending on route of infection, and for *Chabertia ovina* is about 40-50 days.

Nematode eggs are generally resistant to climatic changes, but newly hatched first and second stage larvae (L1 and L2) are bacterial feeders and remain within the faecal pellet or dung pats where they are susceptible to adverse conditions. Non-feeding infective larvae (L3) are more resistant but survival on pasture is dependent on moisture and temperature.

Some infective larvae can survive low temperatures and can over-winter on pasture, but *H contortus* is usually restricted to warmer subtropical and tropical areas with high humidity and requires temperatures above 15°C for development of eggs.

Nematodirus species in Australia and New Zealand are primarily *Nematodirus helvetianus* (cattle) and *Nematodirus filicollis* or *Nematodirus spathiger* (sheep) where the L3 develops within the egg before hatching. Unlike *Nematodirus battus* in Europe, which requires a warm spell following an extended cold period before the eggs are infective, such as occurs in spring to over-wintered eggs, no such trigger is required in Australia. *Nematodirus* sp eggs are relatively resistant to drying and nematodiosis is usually seen as clinical disease in young sheep following the autumn seasonal break of rainstorms after a dry period or drought, or in irrigation areas such as the Murrumbidgee Irrigation Area of southern NSW.

Inhibition (arrest) of larval development occurs in some of the Trichostrongylids, notably *H contortus*, and *Tel circumcincta* in sheep and *O ostertagi* in cattle, either at the early or late L4 stage in the abomasum. In some cases this is related to exposure of L3 on pasture to decreased temperatures in late autumn. Release of inhibition can be triggered without the uptake of fresh infective larvae, by decreased immune status of the host in mid-winter leading to type II ostertagiasis. This is characterised by sudden weight loss and diarrhoea as synchronised larval development re-commences.

Occurrence and Distribution

There are major regional and climatic differences in the distribution of the GIN in Australia and New Zealand, related to the distribution of cattle and sheep and the differing methods of husbandry. Detailed descriptions are available for Australia^{2,3,4,5} and for New Zealand^{6,7,8} with a cattle parasite atlas⁹, but are beyond the scope of this review. *Teladorsagia* spp in small ruminants and *Ostertagia* in cattle tend to be more important in winter and non-seasonal rainfall areas. *Ostertagia ostertagi* is considered to be the most pathogenic cattle nematode in southern Australia and New Zealand. *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus* occur commonly in sheep in Australia and New Zealand.

Sheep

Australia

There are no significant numbers of sheep in the Northern Territory or northern Western Australia. In Queensland sheep are restricted to low rainfall parts of the pastoral zone of western, central and southern Queensland south of the Tropic of Capricorn, where *Haemonchus* is the dominant species. In the north-eastern part of New South Wales (Northern (New England) Tableland region), which is a summer rainfall-dominant area, *H contortus* with some *Trichostrongylus colubriformis* are the two most important parasites. *Oesophagostomum venulosum* is widely distributed but usually only present in low numbers. *Oes columbianum* was believed to have been essentially eliminated from New England and southern Queensland with the introduction of broad spectrum benzimidazole (BZ) anthelmintics in the 1960s. However, recent reports indicate that it is still present and causes financial loss in the processing of cull-for-age ewes and wethers for meat.¹⁰

In non-seasonal or winter-dominant rainfall areas of southern Australia, mixed infections with scour worms *T colubriformis* and *Tel circumcineta* predominate, with small areas of southern NSW having pockets of cold-adapted *H contortus*. *Trichostrongylus vitrinus* is often more common than *T colubriformis* in these areas of Victoria, South Australia and Tasmania.

In Western Australia, the major parasites are *Tel circumcineta* and *T colubriformis* with generally low prevalence of *H contortus*. *Chabertia ovina*, which was once a major pathogen in the so-called “Mediterranean” climate areas of WA and South Australia, is still common in WA but rarely occurs in large numbers. However, larval differentiation in WA indicates *C ovina* can often comprise more than 30% of the worm population. *Oes columbianum* does not occur in WA.

Heavy infections of *Nematodirus* spp can be seen in young sheep under or soon after drought conditions in Australia (south-western NSW, for example) presumably because *Nematodirus* eggs are relatively tolerant to desiccation. Clinical nematodiosis is also not uncommon in young lambs in irrigation areas such as the Riverina area of southern New South Wales.

Bunostomum trigonocephalum is a potentially pathogenic parasite of sheep recorded from all Australian States, but is relatively uncommon and burdens tend to be light and of little consequence.

Dictyocaulus filaria the ‘large lung worm’, *Protostrongylus rufescens* (‘small lung worm’) and *Muellerius capillaris* (‘nodular lung worm’) are found in sheep and goats especially in cool moist climatic areas, but are generally not economically important.

New Zealand^{8,11}

Twenty-nine species of nematodes have been reported from sheep in New Zealand but only five genera are considered economically important pathogens. *Oes columbianum* has been reported only in sheep imported from Australia and is not considered endemic. Abomasal nematodes include *H contortus*, *Tel circumcineta*, other *Ostertagia* species (*O trifurcata*) and *T axei*. In the small intestine *T colubriformis* and *T vitrinus*, *Nematodirus* (*N spathiger* and *N filicollis*) and to a lesser extent *Cooperia curticei* are predominant. Lung worms are infrequently seen.

Climatic variations throughout NZ are relatively small and seasonal conditions are good for development throughout at least part of the year. *Ostertagia* and *Trichostrongylus* are found

throughout the country, with *Haemonchus* and *Cooperia* more common in the North Island. *Nematodirus* is more important in the parts of the South Island. Seasonal patterns of GIN are usually repeatable from year to year, with young sheep being the most susceptible and having mixed infections.

Populations of *Strongyloides* dominate early infections of young lambs, *Nematodirus* spp in late spring, then *Ostertagia* and *H. contortus* and intestinal *Trichostrongylus* in late summer/autumn. *Cooperia* and *T. axei* are usually found in autumn but can survive in sheep over winter and comprise the bulk of infections in the second year of life.

Decline in worm burdens over winter occurs from the development of immunity and results in substantial elimination of worm burdens by the time they are 10-12 months old.

Ewes are not a major source of pasture contamination apart from a period around parturition when periparturient relaxation of resistance results in increased egg output from ewes. Lambs become infected with spring peak of pasture larvae partly derived from the ewes and overwintered L3. Worm burdens build up over summer and a second peak of pasture larvae in autumn from these lambs contributes to the second generation of worms in the weaners.¹² Temperatures around 10°C are the threshold for development of eggs and this affects the seasonal and species development of GIN in different parts of the country.

Cattle

Australia

The distribution of cattle nematodes in Australia is generally related to climate, with *Cooperia*, and *Trichuris* species found in all cattle raising areas.

Ostertagia ostertagi (small brown stomach worm) is probably the most important and pathogenic nematode in cattle in all temperate zones, including the hot dry summer and cold wet winter region of south-western WA. *O. ostertagi* is not reported as endemic in the subtropics or tropical zone of northern Australia, the exception being occasional infections, usually introduced in dairy cattle imported from Victoria, on the Atherton Tablelands district of north Queensland. In Australia, type I infections due to adult worms occur mainly in dairy calves 3-10 months of age and weaned beef calves 6-12 months of age during late winter and early spring. Pre-type II *O. ostertagi* occurs mainly in beef cattle during spring and summer, with inhibited larvae resuming development 4-6 months later in late summer / early autumn.

Outbreaks of type II ostertagiosis caused by massive synchronous development of inhibited larvae with diarrhoea and rapid weight loss may be seen in 18-month-old beef cattle in autumn and in heifers and cows soon after calving.

However, the incidence of type II and other forms of clinical ostertagiosis has tended to decrease with the introduction of anthelmintics with greater efficacy against inhibited and other early stages of parasitic worms. These anthelmintics include the third generation benzimidazole carbamates (fenbendazole, oxfendazole, albendazole, etc), but more particularly the macrocyclic lactones (ivermectin, abamectin, moxidectin, doramectin, eprinomectin), which tend to have consistently high efficacy, especially against inhibited stages, as well as persistent activity against incoming ingested L3s.

Haemonchus placei in cattle is mainly found in the warmer areas, as is the nodule worm *Oesophagostomum radiatum*, which can be a major cause of blood loss and anaemia, particularly

in young weaners. *Bunostomum phlebotomum* (cattle hookworm) is mainly transmitted in the warm wet period (November to March) in coastal south east Queensland and northern NSW to dairy calves, especially those kept in unhygienic conditions and at high stocking densities. This hookworm occurs principally in the proximal small intestine as mixed infections in dairy calves in southern Queensland and NSW.

T colubriformis and *T longispicularis* are recorded in Australian cattle (the latter more so in Western Australia). Small numbers are usually mixed with larger numbers of *Cooperia* spp.

The *Cooperia* species are slightly different in that *C oncophora* is found in temperate areas, whereas this species is replaced by *Cooperia punctata* and *Cooperia pectinata* in the subtropics and tropical areas. *Cooperia punctata*, *C pectinata* and *C oncophora* occur commonly in the proximal half of the small intestine of cattle, with the first two being more pathogenic and occurring together as a complex. *C oncophora* occurs mainly in cooler southern regions of Australia.

Lungworms (*Dictyocaulus viviparus*) are usually an incidental parasite in cattle particularly on the wetter coastal regions from south east Queensland south to western Victoria.

Nematodirus helvetianus, although relatively common in dairy calves in winter rainfall areas of southern Australia, are usually not present in sufficient numbers to be pathogenic.

New Zealand

Limited studies on cattle nematodes were not conducted until the 1960s when post-mortem worm counts of 77 abomasums, 67 small intestines and 26 large intestines from 45 localities in both South and North Islands were conducted.⁶ Twenty-two species were recorded, of which *Cooperia* were found in 100%, *Ostertagia* 97%, *T axei* 82%, *Trichuris* 65%, *Capillaria* 43%, *Oesophagostomum* 39%, *Bunostomum* 12%, *Haemonchus* 9% and *Nematodirus* 8% of cattle. When considering prevalence and numbers, *O ostertagi*, *C oncophora* and *T axei* are the most important. There were no seasonal or geographic differences. Minor species were often sheep species derived from cross-transmission.

Although Brunson⁶ claimed to find *H placei*, Bisset⁷ considered that it was not endemic, as no further specimens had been identified. Lungworms (*D viviparus*) were once common when dedicated small paddocks for pre-weaner calves were prevalent but with changed husbandry practices are no longer regarded as being important.

Clinical disease is principally seen in weaner cattle under one year old. Type II ostertagiosis is the main exception as it occurs sporadically in adult cattle. Clinical signs are loss of appetite and diarrhoea leading to dehydration and weight loss.⁷

Gross Pathology¹³

Parasites of the abomasum

Haemonchus (barbers pole worm)

Haemonchus spp are among the most pathogenic helminth species of ruminants in Australia and New Zealand. Both the developing 4th larval stages (L4s) and adults cause punctiform haemorrhages at sites of feeding on the abomasal mucosa, which may be oedematous. The ingesta may be reddish brown and fluid. Worms may be attached to the mucosa and free in the lumen.

***Teladorsagia/Ostertagia* (small brown stomach worm)**

Developing larvae of *Tel circumcincta* in the abomasum of sheep cause necrosis of the glandular epithelium and replacement of acid producing cells with mucus producing cells.

In cattle type 1 *O ostertagi* infections are composed almost entirely of adult worms resulting from the majority of ingested larvae developing normally to adults in 18-20 days. White, raised, umbilicated nodules (containing developing L4 worms) occur mainly in the fundic mucosa. As the larvae develop and emerge from gastric glands, hyperplasia of gastric epithelium may cause enlargement and coalescing of nodules, the mucosa classically referred to as having a 'Morocco leather' appearance. Mucosal congestion and oedema is also evident, with thickening of abomasal folds.

Pre-type II infections consist of large numbers of inhibited (hypobiotic) early L4s in the gastric glands with minimal tissue reaction and few clinical signs apart possibly from illthrift.

Type II infections consist of adult worms arising from simultaneous maturation of many inhibited early L4s, with glandular hyperplasia, loss of gastric structure, abomasitis, impairment of protein digestion, and leakage of plasma proteins especially albumin into the gut lumen. The mucosa appears thickened and oedematous.

***Trichostrongylus axei* (stomach hair worm)**

T axei occurs commonly in ruminants, often in association with *Ostertagia*, but appears to be relatively non-pathogenic.

In heavy infections, aggregations of worms occur mainly in the fundus, with localised hyperaemia progressing to catarrhal inflammation with white raised circular plaques. Heavy burdens (40-70,000 or more worms) may exacerbate *Ostertagia*-associated gastritis and accompanying clinical signs.

Parasites of the intestines

Small intestine

Intestinal *Trichostrongylus* spp (black scour worms)

Trichostrongylus spp cause similar damage to the anterior small intestine. Developing larval stages in the epithelium result in thickening of the lamina propria, oedema and inflammatory infiltration, with protein-losing enteropathy. Severe villous atrophy, with flattening of the mucosa and irregular masses and ridges may eventuate.

Sub-optimal nutrition exacerbates pathogenicity.

T colubriformis and *T longispicularis* are relatively harmless to young cattle.

***Cooperia* spp**

Gross pathological changes in the proximal half of the small intestine associated with *Cooperia* spp are typical of those of parasitic gastroenteritis. Mucosal inflammation and thickening, epithelial erosions (with leakage of plasma proteins into the gut lumen) and a profuse mucous exudate may be found at necropsy.

Large worm burdens in cattle often in excess of 500,000 may be acquired over a short period, with inhibited early L4s comprising up to 50% of the population, but even such large numbers are not usually particularly pathogenic on their own.

***Nematodirus* spp (thin-necked intestinal worm)**

N spathiger is a very common parasite of young Australian sheep, and usually relatively non-pathogenic, unlike the situation in New Zealand where this parasite inexplicably has become

more important. Severe villous atrophy with mucous-cell hyperplasia, and necrosis may be associated with early larvae invading the intestinal epithelium.

N. helvetianus occurs commonly but in small numbers in dairy calves, usually mixed with much larger numbers of *Cooperia*. Alone they appear to be of little significance.

***Bunostomum* spp (hookworm)**

Bunostomum trigonocephalum is a potentially pathogenic parasite of sheep. Illthrift and anaemia have been attributed to this parasite in New Zealand.

Bunostomum phlebotomum, in cattle, occurs principally in the proximal small intestine. Worms attach to the mucosa by a large buccal capsule, causing mucosal inflammation, thickening and punctiform haemorrhages.

***Strongyloides* spp (threadworms)**

Strongyloides papillosus eggs are often seen in faecal counts in sheep, but this parasite is of doubtful significance.

Female adults are very small (3-6 mm long) and parasitise the proximal small intestine, deep in the mucosal crypts, and so are usually overlooked on necropsy. Heavy infections may cause extensive erosion of duodenum and jejunum with only the muscularis layer remaining intact, and fluid gut contents.

S. papillosus can infect animals by ingestion, skin penetration (in wet conditions) causing an erythematous reaction and urticaria and through the milk of lactating ewes.

Parasites of the large intestine

***Oesophagostomum* spp (nodule and large bowel worms)**

Histotrophic phases of larval stages (L3/L4) of *O. columbianum* cause caseous nodules 0.5-1 cm diameter (histologically eosinophilic granulomata) in small intestines and colon, although small intestinal nodules may be more 'gritty' than 'cheesy'. Nodules can also be found in the lung, liver, mesentery and mesenteric lymph node.

O. venulosum is a mildly or non-pathogenic species. *O. venulosum*-associated nodules occur infrequently, are small, and occur mainly in the caecum and colon.

Oesophagostomum radiatum (nodular worm) and *O. venulosum* occur in cattle; the former being the significant parasite and the most frequently encountered large bowel parasite of cattle. *O. radiatum* adults (14-22 mm long) are whitish and found in thick mucus in the caecum and proximal colon. Numerous nodular lesions, 3-6 mm diameter and resulting from the histotrophic phase, appear scattered on the serosa of the small intestine and to a lesser extent the caecum and colon. In heavy infections, the caecal and proximal colonic mucosa is congested, oedematous and thickened with excessive amounts of turbid mucus. As with *O. venulosum* in small ruminants, this parasite in cattle is relatively harmless. There is a histotrophic phase but little nodule formation.

***Chabertia ovina* (large-mouthed bowel worm)**

This parasite widely occurs in sheep, cattle and goats, usually in low numbers. It has little pathogenic significance in cattle but occasionally causes clinical disease in small ruminants.

Like *Oesophagostomum*, there is a histotrophic phase, with L3s entering the wall of the small intestine, re-emerging and then maturing in the caecum and proximal spiral colon. Adults take a

plug of mucosa into the buccal cavity, causing punctiform haemorrhages, protein loss and oedema.

***Trichuris* spp (whipworms)**

The most common species in Australian cattle, sheep and goats are *T ovis* and *T globulosa*. They attach by their filamentous anterior ends to the mucosa. *Trichuris* spp are considered harmless except in very heavy infections (for example, large soil intake by grazing animals in drought) in which case there may be a sub-acute typhlocolitis, diarrhoea and illthrift.

Diagnostic Tests

For a review of diagnostic techniques in cattle, see Smeal.⁵ Increased serum pepsinogen (see previous ASDT Pepsinogen activity determination in serum and plasma by DI Paynter) levels due to abomasal mucosa damage may be associated with larval stages of *O ostertagi* in cattle, especially in adults, but are insufficiently specific and so should be used with caution and at best only as a herd test.

Faecal egg counts and larval cultures

The tests most commonly employed for the in vivo diagnosis of gastrointestinal and pulmonary nematode infections in ruminants are faecal worm egg counts (FECs), preferably with speciation by way of larval culture and differentiation.

Flotation techniques using highly dense saturated solutions (salt, sugar, magnesium sulphate etc.), with or without centrifugation are well documented, simple and effective for even the most basic laboratories.

FECs do not always correlate well with the number of adult worms present, particularly in cattle over 9-12 months. 'Diagnostic drenching' may be a useful test in such cases. FECs may also be low or zero in the presence of large numbers of immature worms. An example of this may be seen with acute *Nematodirus* infections in young sheep after drought-breaking rains in south-western NSW. Type 2 ostertagiosis in cattle is another example. Also, *Teladorsagia* burdens in small ruminants do not always correlate well with FECs.

Ova of the ruminant nematodes *Nematodirus*, *Strongyloides* and *Trichuris* are distinctive, but differentiation of the more common trichostrongyloid species requires examination of third-stage larvae produced by faecal cultures.

Total worm counts

Necropsy is the most direct method to diagnose gastrointestinal (GI) parasitism. *Haemonchus*, *Bunostomum*, *Oesophagostomum*, *Trichuris* and *Chabertia* adults can be easily seen. However, important infections with *Ostertagia*, *Trichostrongylus*, *Cooperia* and *Nematodirus* are difficult to see (particularly those species spread over metres of intestine), except by their movements in fluid ingesta. These smaller nematodes can be better seen in GI tract washings, particularly against a white background, by staining for 5 minutes with strong iodine solution followed by decolourising background gut material with 5% sodium thiosulphate ('hypo'). Unfortunately the relatively high cost of total worm counts in the laboratory often precludes the use of this test, but it is highly recommended to confirm uncertain diagnoses or where anthelmintic resistance is of major concern.

Mixed infections are the rule. Counts of samples of convenient size (5 or 10% of total ingesta volume), and morphological identification of larval and adult stages of standardised numbers of

worms are required. Digestion of the mucosa of different regions of the gut may be necessary to detect presence of inhibited larval stages.

Anthelmintic resistance testing

Faecal egg count reduction tests (FECRT)

The efficacy of different chemical classes of anthelmintics against the worm populations on individual properties needs to be tested to ensure that only effective drenches are used for control programmes. Widespread and severe resistance to the early broad-spectrum groups of the benzimidazole (BZ), imidothiazole/levamisole (LEV) and BZ/LEV combinations are now the rule in the most important nematode species. Increasingly, resistance to macrocyclic lactone/milbemycin (ML) anthelmintics is being reported, in both sheep/goats and more recently in cattle.

The FECRT (also known as Drenchtest) relies on the principle that faecal worm egg output is indicative of adult worm infections, and that treatment will reduce or eliminate egg-laying worms. Groups of animals sampled at and after treatment are sometimes compared with untreated groups. Allowance must be made in the sampling regimen for likelihood of temporary suppression of egg laying without killing of adult worms by known chemical groups (BZ or ML).

Combinations and various dose rates of drenches can be tested. Testing requires considerable on-farm work to allocate animals to groups, treatment, and faecal sampling.

Improvement to the method is possible if controlled slaughter and total worm counts can be conducted, and may be required for regulatory purposes for registration of anthelmintics or for research purposes.

Larval development assay (LDA)

This is an in-vitro test in a 96-well plate format, which relies on the ability of resistant nematode eggs to hatch and develop to third stage (L3) larvae in the presence of increasing doses of major anthelmintic classes. It has been developed as both an in-house test and commercially as the DrenchRite™. Major limitations of the test are that it is standardised only for BZ, LEV, and BZ/LEV combinations and in certain hands is able to detect susceptibility to MLs. Generally, however, it is not definitive in confirming or quantifying ML resistance. It cannot be used for certain narrow spectrum drenches such as closantel, naphthalophos, or modern combinations including triple and quadruple combination (for example, Triton™, Hatrick®, Q-Drench®).

The advantage is that it is 'farmer friendly', requiring simple bulk sampling, but is laboratory intensive. It is often the first choice if a farmer has no idea of the drench resistance status of his animals. The LDA is not validated for cattle nematodes.

The Egg Hatch Test (EHT) is applicable only for detection BZ resistance, which is so common and severe in Australia and New Zealand that the EHT is redundant, although it is still being advocated in Europe and the USA.¹⁴

Serological and Molecular Biology tests

Because nearly all sheep and cattle are infected with a mixture of nematode species, serological tests, especially ELISA, have not been widely used for diagnosis of GIN infections. An exception is in cases of ostertagiosis, particularly pre-type II. Cross-reactions between trichostrongyle species are common. An antibody detection ELISA for *O. ostertagi* antibodies in milk (SVANOVIR® *O.ostertagi*-Ab ELISA, Svanova Biotech Ab, Uppsala, Sweden;

www.svanova.com) has recently been developed in collaboration with the Faculty of Veterinary Medicine, Ghent University, Belgium. It has not been validated for use in Australia.

Antibody ELISAs are used primarily for research purposes to monitor developing immunity, but persistent antibody production is a common limitation. Attempts to develop species-specific faecal antigen detection methods for a more rapid differentiation of species than is possible in the one week it takes to do larval culture and differentiation, and speciation of eggs using antibody-labelled lectins await results.

Variations of polymerase chain reactions (PCR) are at present not commonly used for species diagnosis but there is increasing interest in PCR for strain characterisation/genomics and rapid identification of anthelmintic resistant isolates from individual worms (even individual L3). At present only the *B*-tubulin mutation in BZ-resistant trichostrongyles is clearly defined.

Part 2. Test Methods

Introduction

Earlier versions of this document were predicated on the hypothesis that the ability of sheep nematodes to develop resistance to anthelmintics was a likely eventuality, and early programmes to control nematodes were based on the application of anthelmintic to sheep populations.

We now know, some 24 years after the first publication of the article in this series¹⁵, that the heavy reliance on treatments with anthelmintics has indeed resulted in worms in high proportions of the Australian sheep flock being resistant to one, two or even three drench classes. Although helminth parasites of livestock do not usually cause the dramatic mortalities produced by many viral and bacterial diseases, the adverse effect of worms on production is probably even greater, although often hidden or insidious.

Although resistance to anthelmintics is not yet as common or important in cattle as in sheep, there is increasing evidence from parts of the world where cattle are given numerous drenches, such as barley beef in the UK and Europe or dairy beef system in New Zealand, that resistance is present to a measurable extent. It is likely that similar findings will occur in Australia as management practices change.

Most of the diagnostic techniques described in the following sections are based on classical parasitological methods. A number of more recent techniques, such as ELISA and some molecular biology methods, have been included to indicate the direction in which some laboratories are moving. At the present time, these tests would not be considered to be standardised or widely used as routine diagnostic tests. Recent reviews should be consulted.^{16,17}

This ANZSDP has changed the emphasis from testing for resistance to anthelmintics to emphasise basic techniques to diagnose worm parasites in sheep, goats, South American camelids (especially alpaca) and cattle.

Alpaca have been included as they are now a not-inconsiderable industry (approximately 40,000 head in Australia according to the Australian Alpaca Association Newsletter 2003) susceptible to both sheep and cattle parasites. Although they have particular dunging habits, such as the use of 'latrine' areas, which reduces their exposure to reinfection from other alpaca, there are opportunities for infection from high contamination when they are used as 'Guardians' of lambing-ewe flocks. Then the peri-parturient relaxation of resistance in the ewes ensures that pastures are heavily contaminated by the high faecal worm egg output of the ewes.

In the mid-1980s, the cost of infections of sheep with helminths was estimated at just under \$5 000 per farm per annum. With the now widespread resistance to anthelmintics, the costs to the sheep industry were estimated in 1995 at \$200m per annum.¹⁸ The Australian Wool Innovation, which is the research and development arm of the Australian wool industry, estimated in 2007 that this cost could rise to \$700m per annum within 7 years, if the current trends of resistance continue and there are no new drug families introduced.¹⁹ Recent information on the economic effects of endemic disease estimated the cost of internal parasites in sheep at \$369 million, and gastrointestinal worms in cattle at \$39 million.²⁰

As resistance to anthelmintics became more obvious and the wool industry changed with the reduction and then abolition of floor prices for wool that resulted in the 'wool stockpile' of the mid-late 1990s, the emphasis of the programmes began to change. They began to incorporate less prescribed or mandatory treatments; with an emphasis on monitoring of faecal worm egg

counts and identification of 'effective' anthelmintics by testing for resistance to anthelmintics. Since 1999 many State agencies have increased the cost of routine parasite monitor testing, which, with the ongoing situation of extensive drought, has resulted in a general down-turn in worm testing. Recent estimates are that less than 5-10% of all farmers regularly (that is, more than twice per year) worm test.²¹ Those who conduct anthelmintic resistance (AR) testing are a minute proportion of these, and those who conduct repeat AR testing at the recommended frequency of every 2 to 3 years are miniscule.

The notable exceptions are in particular regional pockets, such as the New England Tablelands of northern NSW, where there has been a history of concentrated extension services by both government and private veterinarians and consultants.²²

Methods for the diagnosis of parasites based on concentration of serum enzymes (for example, plasma pepsinogen in ostertagiosis) are not included in this ANZSDP, as they are insufficiently specific for particular parasites.

When State-wide or regional programmes to control sheep helminths were first instituted in the late 1980s and early 1990s, they were based on clearly defined drenching at precise points in the animal husbandry calendar. Although lip service was paid to other non-chemical methods of control, such as alternate grazing, pasture management including production of low worm-risk pastures, 'drench and move' and breeding of worm-resistant sheep, in effect most farmers relied heavily on routine and frequent application of anthelmintics.

The current programmes conducted by Australian Departments of Agriculture or Primary Industries are shown in Table 1. The implementation of these programmes has been reviewed to highlight the challenges.²³

The names of the programmes, with the use of phrases such as 'Drench...' or '... kill' suggest the predominance given, at least in the early days of these schemes, to heavy reliance on chemical control. Several of the smaller States that have diverted diagnostic testing to private laboratories no longer operate officially sanctioned programmes. In others, the programmes exist only in the form of publications advocating integrated parasite management (IPM). Full details of these programmes can be obtained from the website of the Australian Wool Innovation/Australian Sheep Industry Collaborative Research Centre sponsored WormBoss at <http://www.wormboss.com.au> and the individual State government agencies.

Control programmes for parasites in goats, alpaca and cattle are usually designed for specific local areas by individual veterinarians (government or private) who have a personal interest in parasite control.

Table 1 Australian Regional Sheep Worm Control Programmes 2008

Name	State	Region	Climate *	Current status
DrenchPlan	NSW	Central and Southern Tablelands, SW Slopes and Riverina	Non-seasonal to Winter dominant rainfall, 200-600 mm pa.	Active
West Worm	NSW	NW Slopes and Plains	Summer dominant rainfall, 200-400 mm pa	Active
Far West Worm	NSW	Pastoral regions of Upper and Lower Western Division	<200 mm rain pa	Active
WormKill	NSW	New England and Northern Tablelands	Summer dominant rainfall 400-900 mm pa	Active
WormWise	WA	Central West, Central Wheat Belt, Great Southern, South West	Winter rainfall (Mediterranean), 300-900 mm pa.	Active
Worm Plan	VIC	Whole State	Winter dominant rainfall, 300-900 mm pa.	Inactive
WormCheck	SA	Agricultural zones	Mediterranean (winter dominant) rainfall, 200-600 mm pa.	Inactive
WormPlan	TAS	Whole State	Winter rainfall, 300-1200 mm pa.	Inactive
WormBuster	QLD	Eastern Maranoa and Darling Downs in SE Qld	Summer dominant rainfall, 300-900 mm pa.	Active

* Rainfall data based on the most recent Australian Rainfall Analysis publication of the Bureau of Meteorology for the period 1 January to 31 December 2006, issued 01/01/2007 at <http://www.bom.gov.au>

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 McMaster slide (see Appendix 2). Direct extrapolation of the number of worms to be found in the gut from the calculation of eggs per gram (epg) of faeces is only approximate. It is more closely correlated in sheep than cattle. In the latter, the relationship is reasonable in young non-immune calves and weaners, but not in older adult cattle (*FECPAK*[™] update on cattle worms, see below). Egg production is influenced by many factors, for example, genetic variations both within and between breeds of hosts, immunity of the host, species of worm, maturity of worms, season of the year and stage of pregnancy or lactation.

Egg counts can give valuable information on existing (adult) worm burdens and larval paddock populations if samples are taken just before drenching, and on anthelmintic efficacy if faecal samples are taken 10-14 days after drenching.

The Modified McMaster Method

This method is based on the McMaster Method²⁴ and uses a Whitlock Universal (4 x 0.5 mL), McMaster (3 x 0.3 mL) or Paracytometer (2 x 0.6 mL) slide. It is the standard procedure adopted for egg counting individual animals. 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 faecal egg count reduction test (FECRT) samples as a correct randomisation of animals will allow for variability in faecal consistency.

A recent publication has revisited the need for assessment of faecal moisture content to modify worm egg counts.²⁵ This may be important for selection of animals when breeding for worm resistance.

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 and roughly break up the pellets with a pair of forceps. Allow the broken pellets to soak, covered, for one hour or refrigerate overnight.

Add 47.5 mL of flotation fluid, usually saturated sodium chloride solution (specific gravity 1.20) to each jar. Some laboratories use saturated magnesium sulphate or saturated sugar (see Appendix 1)

Mix with a mechanical or air-powered mixer or a hand-held kitchen mixer until the faeces are broken up and well dispersed.

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 70% ethanol or methylated spirits will disperse any bubbles.

Pipette a sample from the centre of the suspension into two chambers of the counting slide. Note well the following points:

- Drain pipette before collecting the sample
- Lightly bounce the pipette while filling to prevent blockage of sieve by fibre.
- Tilt the mixture to allow maximum filling of the pipette.

- Dab the end of the pipette with tissues after filling to remove any bubbles from the surface of the mixture.
- Ensure that the pipette is held horizontally and fill the chamber in one action without producing bubbles. Allow 1 to several min for the eggs to float up under the glass before counting.

Count the eggs in the chambers using x40 (Not x 40 objective lens) to x100 final magnification. Count all the eggs within the double line boundaries.

Calculate the epg of faeces by multiplying the number of eggs counted by the total volume (50 mL) divided by the volume counted times the weight of faeces (Table 2).

Table 2: 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) ¹	0.5 mL	1.0 mL
Weight faeces	2 g	2 g
Multiplication factor	x50	x25

¹ Whitlock Universal chamber

If other counting slides are used, make appropriate adjustments for volume when calculating multiplication factors. For example, if using a Paracytometer slide, make up to 60 mL total volume and the count per 0.6 mL chamber is multiplied by 50. 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.²⁶ *Nematodirus* eggs should be counted separately from strongyle eggs. Coccidial oocysts can also be counted or estimated using a semi-quantitative scale.

Usually 10 animals per group are sampled and the faeces are processed individually, resulting in 10 egg counts (two batches of 5 are counted in the NSW DPI and QDPI&F “Basic Wormtest”).

Egg recovery rate with McMaster technique is approximately 70%, but is not usually corrected.

Composite Bulking Technique

A variation of the modified McMaster method²⁷ uses a bulking technique prior to mixing for the batch processing of large numbers of samples when predominantly *Haemonchus/Trichostrongylus* are present and for flock monitoring, but not for FECR tests as high counts in one animal can lead to incorrect conclusions on resistance status. 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.

Weigh 0.5 g from each of five samples into the jar to make a 2.5 g sample (A). Repeat the procedure for the next five samples (B) from the group of 10 samples.

Process separately each of the two composite samples as for the McMaster method above. Average the count of two chambers for each of A and B.

The multiplication factor is x40 of the mean of A and B to express the results as egg. If fewer than 10 samples are to be processed the table below should be consulted for the multiplication factors (Table 3).

Table 3: Multiplication factor based on pooling 0.5 g samples and reading total eggs in one chamber of volume 0.5 mL

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

NB 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.

Centrifugation Method

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

Weigh 1.5 g of faeces from each sample into a 60 mL container.

Add 28.5 mL of clean tap water from a dispenser to each sample. Allow to soak for from several minutes to one hour to soften the faeces.

Homogenise each sample using a laboratory stirrer or shake with glass beads.

Pass the homogenised samples through a small household tea strainer mounted over a bowl of suitable size to collect the liquid.

Swirl to mix the liquid, then immediately pour into a 15 mL numbered centrifuge tube. Excess liquid can flow over the tube once it is full.

Centrifuge at approx 250 g for two minutes to produce a plug of debris containing the eggs.

Pour off the supernatant carefully or remove it with a water-driven suction pipette.

Add saturated sodium chloride solution up to the 10 mL mark. Resuspend the plug by repeated inversions of the tube or use a rotary mixer. Fill the tube up to the 15 mL mark with more saturated sodium chloride solution and mix thoroughly using inversion or a Pasteur pipette.

After mixing the faecal suspension, fill the chamber of a Universal Whitlock slide (0.5 mL in each chamber). Count all eggs within the first two lines of the chamber. The multiplication factor is 100. If few eggs are present, count chambers 1 and 2 to give a sensitivity of 20 egg.

Wash the stirrer, the mixing container, the sieve and the collecting bowl between samples. The method is claimed to have 100% predictive value (no false negatives).

Centrifugation method (after Egwang and Slocombe²⁸)

This Cornell-Wisconsin centrifugal flotation technique has been evaluated for bovine faeces with low numbers of nematode eggs.²⁸ It is the required method for faecal examination of sheep

or lamoids for export to New Zealand (see NZ Biosecurity: *Import Health Standard for Importation of Alpacas and Llamas into New Zealand from Australia*, issued 8 May 2006 retrieved from <http://www.biosecurity.govt.nz/files//imports/animals/standards/lamaniic.aus.pdf>).

Optimal conditions are as follows:

Mix 5 g bovine faeces with 12 mL water and strain mixture through a domestic tea strainer. Retain liquid. Rinse container and strain washings.

Press wet faecal material to obtain as much liquid as possible, then discard the faecal residue. Strained liquid is transferred to 15 mL centrifuge tube and centrifuged at approximately 264 g for 3 min (swinging bucket rotor essential).

Discard supernatant, being careful not to displace fine sediment on top of residue.

Half fill tube with saturated sugar solution (SG 1.27) and mix with an applicator stick. Add equal volume of saturated sugar solution, mix again and add further sugar solution using a dropper (or Pasteur pipette) to completely fill. **Caution:** Do not allow material to overflow.

Place in centrifuge and carefully place a 22 x 22 mm cover slip on the meniscus.

Centrifuge at 264 g for 5 min.

Remove coverslip by lifting off vertically. Add drop of sucrose to the glass slide and place the coverslip on the slide, being careful to exclude any air bubbles. Examine at x40 magnification.

Count all eggs under cover slip. Egg count is divided by 5 to determine egg.

The egg can be multiplied by 1.6 as the recovery rate is only 62.5%.²⁸

The method is not suitable for large numbers of eggs, when less than 5 g of faeces should be used as it counts the total number of eggs, not just a small proportion.

The method is claimed to have 100% predictive value (no false negatives).

FECPAKTM

This method is a commercial kit for faecal egg counts, primarily designed for 'Do It Yourself' (DIY) egg counts. The principle is that faeces (especially from cattle) have low counts and displacement method into zip-lock plastic bags is used in place of weighing out into jars. The method has been validated against other standard techniques, such as centrifugation and McMaster counts by laboratories in UK, Europe, Ireland and New Zealand. Generally the method is as sensitive as others but may have greater variability. Details are available from the FECPAK website at www.fecpak.com/rp-validation.php and have been published.¹⁴

Cytochemistry of Nematode Eggs

Selective staining of sheep trichostrongyle eggs with fluorochrome-labelled agglutinins (lectins), for species identification has been tested with limited success by several authors.^{29,30}

Haemonchus contortus stains strongly with peanut agglutinin (PNA), *Trichostrongylus* spp strongly with *Maclura pomiera* lectin (MPA), while *Teladorsagia circumcincta* was weakly positive for PNA, MPA and concanavalin A (ConA). *N. spathiger* was only weakly positive for all 4 lectins. Mixed genera produced by artificially mixing various proportions of the three main species (*Haemonchus*, *Trichostrongylus* and *Teladorsagia*) could be identified but in field mixtures, when compared with larval culture and differentiation, there was good agreement in estimating prevalence of *Haemonchus* and *Trichostrongylus*, $r^2 = 0.618$ and 0.651 respectively, but poor for *Teladorsagia* ($r^2 = 0.042$).³⁰

Samples fixed in 4% formalin equalled fresh samples in their ability to bind lectins. Specimens can be examined by fluorescent microscopy or flow cytometry.

Flow cytometry is still experimental and cannot be used diagnostically as validation data are not available.

Reagents

Fluorescein-isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) coupled lectins (Sigma); peanut agglutinin (PNA), concanavalin A (Con A), *Ricinus communis* agglutinin 120 (RCA) and *Maclura pomifera* agglutinin (MCA) suspended at 1 µg/µL in phosphate buffer saline (PBS).

Isolation and purification of eggs (adapted from Colditz et al³⁰)

1. To a 250 mL jar, add all of faecal sample (at least 20 g) to the nearest gram.
2. Add 5 mL of distilled water for every gram of faeces present.
3. Place sample in refrigerator for approximately one hour to allow faeces to soften and to prevent eggs from embryonating.
4. Blend faeces thoroughly.
5. Remove 36 mL sub-sample (equivalent to 6 g of faeces) to new jar.
6. Add 144 mL saturated sodium chloride salt solution to faecal matter, giving final proportions of two parts water to one part saturated salt solution.
7. Insert specially made 142 µm sieve cage in sample.
8. Immediately withdraw 40 mL (equivalent of 1.3 g faeces) and add to 50 mL conical-based centrifuge tube.
9. Centrifuge tube at 2000 g for 10 min.
10. Transfer supernatant (containing the eggs) to a new tube, discard the pellet of faecal debris.
11. Repeat steps 9 and 10
12. To the supernatant add 10 mL distilled water and mix.
13. Centrifuge at 2000 g for 5 min to pellet eggs.
14. Discard supernatant and resuspend eggs in ~200 µL PBS for immediate staining, or in 10% formalin in PBS for storage at 4°C.

Staining eggs for fluorescent microscopy

1. For stored samples, wash once in PBS to remove formalin then centrifuge at 1500 g for 5 min.
2. Remove supernatant with transfer pipette, leaving 100-200 µL above pellet.
3. Resuspend eggs in remaining liquid.
4. Add 100 µL of 1 mg/mL TRITC-labelled PNA.
5. Add 100 µL of 1 mg/mL FITC-labelled MPA (step can be discounted to save expense of additional reagents, and total egg counts made using normal brightfield illumination).
6. Mix and incubate at ambient temperature in the dark for ~80 min. (Staining intensity plateaus after this time).
7. Wash once in 20 mL PBS, centrifuging at 1500 g for 5 min.
8. Remove supernatant with transfer pipette leaving ~100µL.
9. Resuspend pellet.
10. Place 15-20 µL on a glass slide and add coverslip.
11. Observe under incandescent light until eggs are observed, and then view under UV light through red (TRITC) and green (FITC) filters.

For flow cytometry examination, eggs should be separated from faeces by bulk separation methods, using filtration of faecal slurry through 150 µm mesh, multiple sedimentation and flotation in saturated NaCl.³⁰

Stain: To approximately 10 000 eggs, incubate with 50 µg lectin in 200 µL at RT in dark for 60 min. Wash twice with 2 mL PBS by centrifugation at 1500 g and resuspend in 200 µL PBS. Examine eggs by microscopy or use of flow cytometer (see below).

Flow Cytometry for Faecal Egg Counting

This method has been developed by CSIRO Livestock Industries³⁰ to count and identify nematode species by using fluorescent dye tags attached to lectins or antibodies developed against eggs of individual species of sheep nematodes. The fluorescent tags are not needed for total egg counting, but for species identification.

A major problem with the counting technique is the difficulties due to blockage of the cytometer probe orifice by faecal debris even when the samples have been extensively sieved. At this stage, the technique is not cost effective due to the very high cost of flow cytometers (tens of thousands of dollars).

Faecal Culture and Identification of Nematode Larvae

The value of a faecal egg count is increased if the genera of worms present can be identified. While most strongyle eggs are similar in shape and size and not readily identified, infective (third stage (L3)) larvae are morphologically distinct and more readily differentiated.

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

Setting up the Culture

For an individual culture, transfer about 20 g of faecal material to a culture bottle (jar). A 250 mL glass or disposable polystyrene 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 (jar) to make a 30 g sample.

Add 20 mL of water. Use more water for larger amounts of faeces. Mix thoroughly (for example, with a hand-held kitchen mixer). Add 5 g vermiculite No 3 (medium grade). Mix lightly with spatula. Do not pack the mixture. Alternately, mix faecal material in a clean mortar. Add vermiculite and some water. Mix with a (plastic) gloved hand or pestle to give crumbly mixture. Transfer to a labelled culture jar. Rinse down the sides of the culture bottle with a small amount of water. Do not pack the mixture. NB The moisture content of a culture is important. High moisture content may lead to massive fungal growth (can be inhibited by stirring during the first three days of culture³¹, or spray of 1 % sodium carbonate) at the expense of larval survival. If faeces are dry, use less vermiculite.

Place the lid (minus wad if applicable) on the bottle, turn lightly but do not seal. Incubate the jar/bottle at 25-27°C in the dark for seven days.

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. NB Some laboratories use a Baermann funnel to separate larvae.

Larvae can now be stored for several weeks at 4-10°C if necessary.

Reading the Culture

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.

Mix larvae, pipette a drop of the suspension onto a microscope slide. Add one drop of parasitological iodine (see Appendix 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.

Count 100-200 larvae, and differentiate into species (see Figure 1, Table 4 and Figures 2a and 2b). Express results as a percentage for each species.

Figure1: Key to identification of the 3rd stage larvae of some common gastro-intestinal nematodes of sheep and cattle (modified from MAFF³¹)

1.	Oesophagus rhabditiform Oesophagus not rhabditiform	Free living nematode 2
2.	Without sheath, oesophagus nearly half length of body With sheath, oesophagus less than ¼ the length of the body	<i>Strongyloides</i> 3
3.	Tail of sheath short or of medium length Tail of sheath very long	4 7
4.	Two refractile bodies or a bright transverse band visible between buccal capsule and oesophagus Refractile bodies or band absent	<i>Cooperia</i> 5
5.	Slender larva, tail of sheath of medium length tapering to a point and often kinked Tail of sheath very short, conical	<i>Haemonchus</i> 6
6.	Larva of medium size or large with distinct rounded tail Small larva, tail bearing one or two tuberosities or indistinctly rounded	<i>Teladorsagia (Ostertagia)</i> <i>Trichostrongylus</i>
7.	Very large larva, 8 gut cells, tail notched, bilobed or trilobed Larva of medium size, 16-24 pentagonal gut cells, lumen of gut wavy Larva of medium size, 24-32 square gut cells, lumen of gut straight Very small larva with 16 gut cells	<i>Nematodirus</i> <i>Oesophagostomum</i> <i>Chabertia</i> <i>Bunostomum</i>

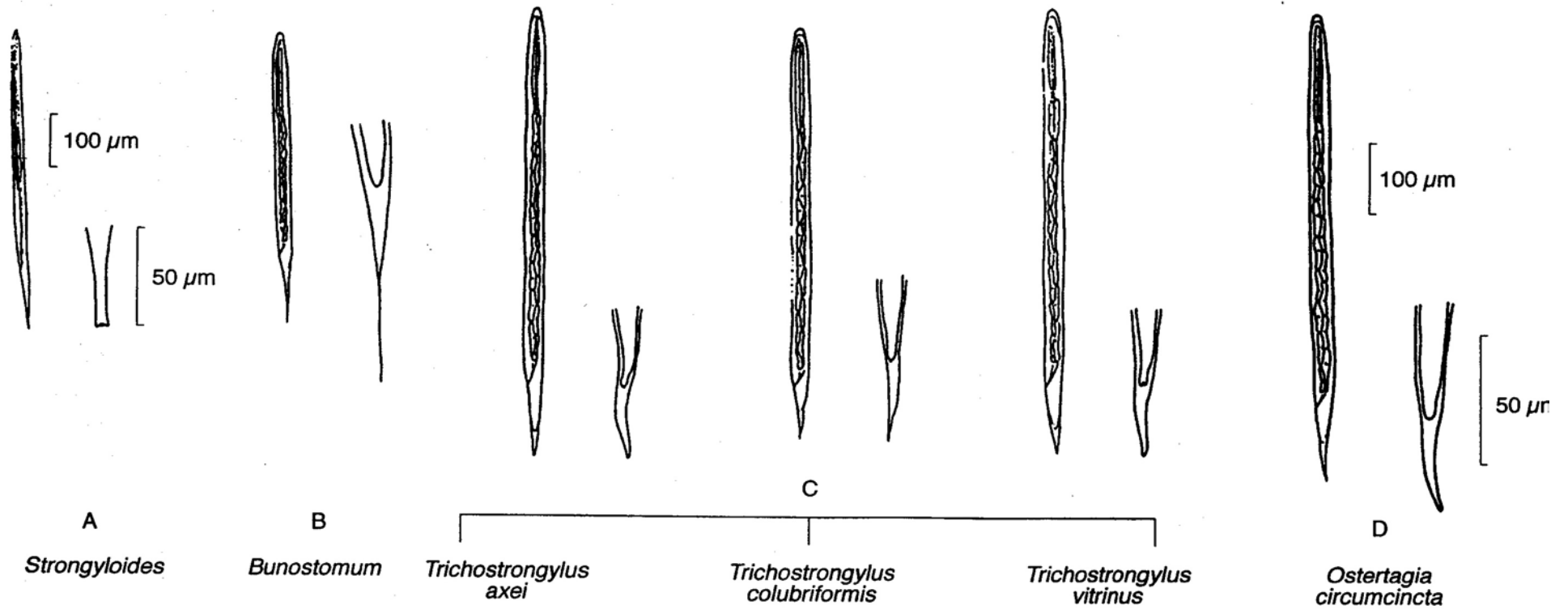


Figure 2a: Infective larvae of parasitic nematodes of sheep (MAFF³²)

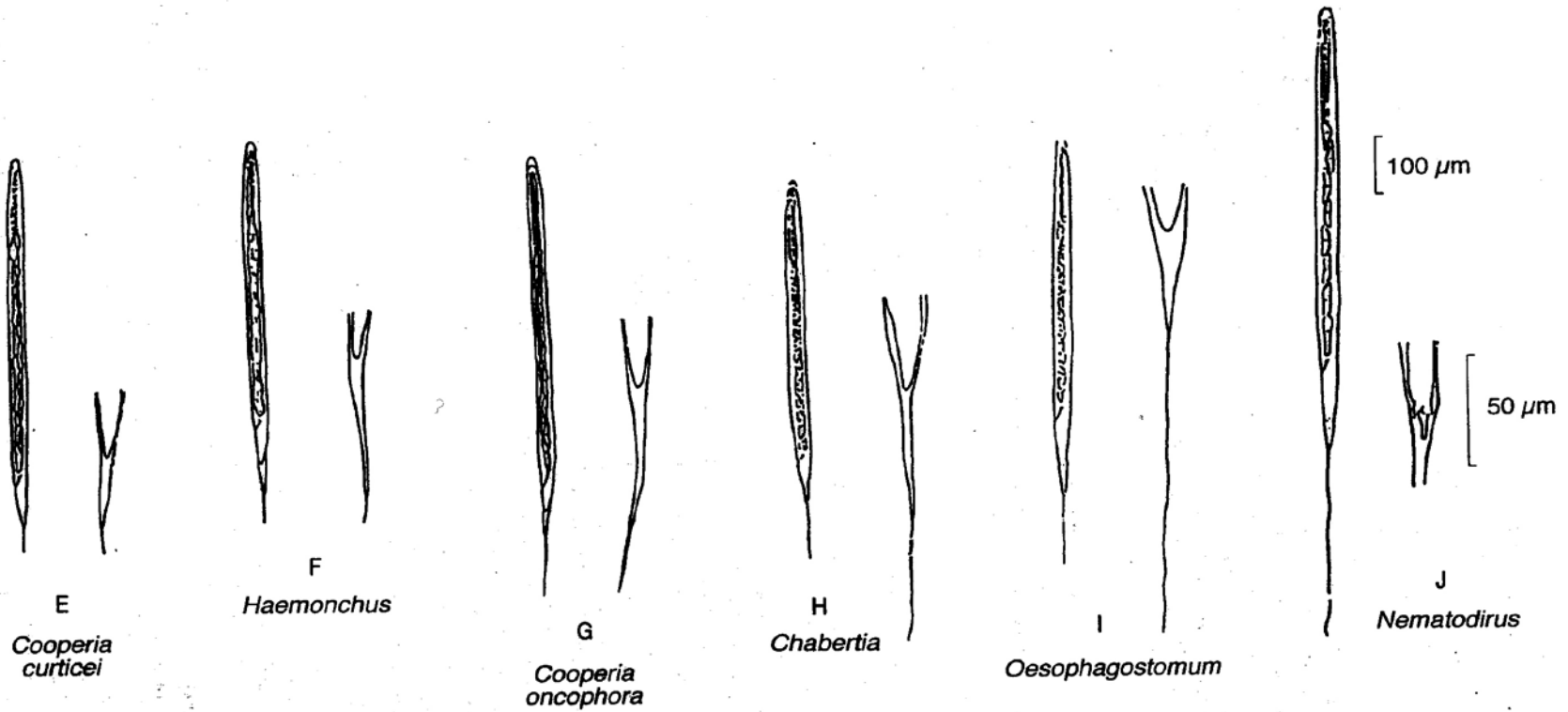


Figure 2b: Infective larvae of parasitic nematodes of sheep (MAFF³²)

**Table 4: Measurement of infective nematode larvae of sheep
(adapted from Dikmans and Andrews³³)[#]**

<i>Length, end of larva to end of sheath (µm)</i>	<i>Species, with range of total length (µm)</i>	<i>Key to Figure 2</i>	<i>Other differing features</i> ^{34, 35, 36}
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 tails sheath. <i>T colubriformis</i> has 2-3 tubercles [@] on tail of larva <i>T vitrinus</i> has one tubercle on tail [@] <i>T axei</i> , no tubercles on tail, tapered head, shoulder
	<i>Teladorsagia (Ostertagia)</i> 700-910	D	<i>Teladorsagia</i> has no tubercle on tail [@] Longer, conical, 'finger-like' sheath, head with distinct shoulder
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 at 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 tails. Tail of larva is forked.

[#] Strain variations exist around this mean

[@] After exsheathing in sodium hypochlorite (photographic concentration)

Larvae of *Teladorsagia (Ostertagia) circumcincta* and *Trichostrongylus* spp, particularly *T colubriformis* and *T vitrinus* can be identified after exsheathing in sodium hypochlorite (NaOCl) solution (household bleach, 4% available Cl₂). Larvae of *T vitrinus* may measure up to 796 µm.^{34,35,36}

Sedimentation /Emigration Technique for Lungworm Larvae

The modified Baermann technique is used for recovery and counting of lungworm (*Dictyocaulus* spp, *Protostrongylus* spp, *Muellerius* spp) larvae in animal faeces. (For the deer lungworm *Elaphostrongylus cervi*, see the previous ASDT *Elaphostrongylus in deer*, by PJA Presidente³⁷). First stage larvae are motile and emerge from faeces in water and sediment. The method is suitable for fresh and chilled faeces less than 7 days old.

The classical Baermann method uses a funnel that is attached to a rubber tube and clamp. A known amount of faeces (for example 5 g) is wrapped in a small parcel with a single sheet of paper tissue (Kimwipes™ fine grade, lint-free, are preferred as they do not disintegrate in water), and placed on a sieve in the top of the funnel. The funnel is filled with (lukewarm) water to cover the parcel of faeces and the larvae allowed to sediment for several hours at room temperature (or 25-27°C). The settled larvae are removed by tapping off the first 10 mL of sediment, and then centrifuging at 300 g for 2 min.

Larvae are recovered from the sediment and placed on a microscope slide, under a coverslip and examined at x100 magnification.

Identification of larvae is based on the presence of tubercles and tail morphology, and granules in the body of the larvae. Care must be exercised in distinguishing L1 of protostrongylid or dictyocaulid larvae from free-living L3 trichostrongylid larvae, which may have developed if the faecal samples have been delayed in transportation to the laboratory or have not been kept cool. (See Figures and descriptions in references 38, 26 and 31).

Low numbers of larvae are common with deer faeces infected with *Elaphostrongylus* (in New Zealand), so larger quantities of faeces (minimum of 20 g) should be sampled from deer. Detailed requirements for the testing of deer before import into Australia have now been withdrawn as all such imports have been prohibited since 2000.

An alternate method that is claimed to be more effective than the funnel method in the recovery of *Dictyocaulus* (137% more), *M capillaris* (175% more) and *E cervi* (1709% more) (mean recovery 19.9 vs 1.1), is the use of urine sedimentation flask, with a faecal parcel suspended by a skewer.³⁹ Replacement of paper tissue with stockinet resulted in more sediment but no difference in the number of larvae recovered.

McKenna concluded that the flask method was superior because of the better recovery, the ease of use, obviating centrifugation, and lessening likelihood of cross-contamination.³⁹

Total Worm Count - Gastrointestinal 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 sampling technique, and the one described is an example. See Powers *et al*⁴⁰ for further total worm counting procedures.

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 (Endecott Ltd test sieves are available from Crown Scientific) 38, 150, 180, and 200-300 μm

Scoops 50 or 100 mL

Pump for aeration

Formalin 10 per cent (3.7% formaldehyde)

Parasitological iodine (see Appendix 1)

Saturated sodium thiosulphate (photographic hypo) (see Appendix 1)

Necropsy

Deprive animals of food for 24 hours before necropsy.

Isolate the gastrointestinal tract into its component parts as soon as possible after death. Strip off adipose tissue and mesenteric attachments and discard.

Locate and tie off each organ separately with string before excising the tract.

If organs cannot be processed immediately, refrigerate at 2-5°C for up to 12 hours. Place each organ in a separate plastic bag and label with the relevant information, for example, ear tag number, date of slaughter, etc. Alternatively, embalming fluid (see Appendix 1) will preserve the contents but leave the gastrointestinal tract in a pliable condition and able to be 'run' (see Appendix 2).

Worm Recovery

Each organ is processed separately.

Abomasum

Open the organ along its greater curvature and spill the contents into a 180 μm sieve. If larval parasites are present, a 38 μm sieve should be placed under the 180 μm 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 (CH₂O) to give a final concentration of 5% formalin. Some laboratories prefer 10% formalin.

Refrigerate at 2-5°C the abomasal wall for digestion (see below) if infection with histotrophic stages (tissue invading) is suspected.

Small Intestine

Most worms are found in the upper half of the small intestine.

Cut open the small intestine, squeeze the contents off between thumb and forefinger and collect by washing onto a 150 μm sieve. Alternatively, a 'gut runner' (see Appendix 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.

Collect and preserve the contents as for the abomasum. Refrigerate small intestinal wall for digestion if necessary.

Large Intestine

Open the rectum and remove enough faeces for a faecal egg count and culture.

Open the organ onto a tray, wash with tap water and add contents to a 200-300 µm sieve. The species present are large and readily seen.

Digestion Technique for the Recovery of Immature Nematodes

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.

Transfer mucosal scrapings or small intestine sections to a large wide-mouthed jar. Add 1 L of digest liquid (see Appendix 1). Loosely place lid on jar.

Incubate at 40°C for 2-4 hours. Stir frequently. Estimate the end point visually.

Remove tissue; strain the liquid through a 38 µm screen to collect the larvae. Back wash contents with tap water into a container. Add buffered formalin (Appendix 1) to give a final concentration of 5% formalin.

Examine a sample for parasites. (Refer to WAAVP Guidelines⁵³ for sample size).

Alternatively, add washings to the contents from abomasum or small intestine, for counting and identification.

Digest Liquid

Pepsin is available from various chemical suppliers. The potency of different batches varies: use 10 g of 3000 unit/L or 2 g of 150 000 units/L.

Worm Counting

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

Dilute contents and digested remains to 2 L 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 a compressed air line. Adjust the rate of mixing so that none of the contents splashes out. Mixing should be in a criss-cross pattern (N-S, E-W) not in a circular motion.

Remove a 10% sample (WAAVP Guidelines⁵³ recommend collection of two 5% samples with one being counted and the second retained as a backup) and examine for parasites. The sample may be counted in one step or several sub-samples totalling 10%. Use 50 or 100 mL scoops for sub-sampling.

Stain each sample with parasitological iodine for a few minutes. Decolourise with 20% sodium thiosulphate. Inhibited early L4 larvae will decolourise very quickly.

Examine under a dissecting microscope at x15 magnification using a petri dish marked with parallel lines approximately 5 mm apart.

Count and differentiate the worms. The total number present in each organ is calculated from

the dilution factors.

Storage solutions for helminths are listed in Appendix 1. Various techniques for being statistically accurate in the estimate of the number of worms present in an organ have been developed.^{41,42}

Differential Worm Count

Identify the first 100 worms seen in each organ to species and stage. Calculate the number of each species present and its stage as a percentage of the total.

Worms can be cleared in lactophenol (see Appendix 1) for a few hours prior to identification under x100 magnification. This especially allows males to be differentiated to species on the basis of spicule morphology.

Worm Identification

Sheep

The following descriptions are intended only to give a general idea of the genus present.^{43,44} For detailed speciation see Soulsby.²⁶ Details of morphological features, especially bursa and spicules are also found in MAFF.³¹ For immature stages see Douvres.⁴⁵

Abomasum

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

Haemonchus spp 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 in the haemoglobin-filled body cavity. The vulval flap can often be seen.

Teladorsagia (Ostertagia) spp are slender brown worms to about 12 mm, uniform in thickness throughout the length.

Trichostrongylus axei is very small, about 4-5 mm, and tapers markedly to the anterior end.

Small Intestine

The worms commonly present are *Trichostrongylus* spp and *Nematodirus* spp and these can be identified macroscopically on size and in 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 filariform (slender cylindrical without bulbs) 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 anterior end is often thickened with inflations to the cuticle. 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 do not 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 and should have characteristic rhabditiform (double bulbed) oesophagus, while the fourth larval stage of *Nematodirus* has a spine on the blunt tail.

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 *Oesophagostomum columbianum* (sheep) are found mainly in the colon but in heavily infected 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.

Cattle

The general morphology of the species of nematodes found in cattle is similar to those in sheep. In the abomasum *Ostertagia ostertagi* is found in place of *Teladorsagia circumcincta* in sheep; both have a small bursa in the male. Although *H placei* primarily infects cattle and *H contortus* is primarily a parasite of sheep, in some regions they are sympatric. Attempts to differentiate three *H placei* sub-species using discriminate functions with *H placei placei* being found in Australia have been made.⁴⁶

In the intestine *Bunostomum phlebotomum* is more common in cattle than is *B trionocephalum* in sheep. It is a parasite with a preference for warmer climates such as coastal Queensland. It is a small but relatively stout worm with a large globular buccal capsule containing cutting plates and teeth. The anterior is bent dorsally. There are long spicules (3-4 mm) in the male.

Serological and Molecular Biological Diagnostic Techniques

Serological and molecular techniques have been developed for a number of nematode infections in domestic livestock, but are mainly used as research techniques, and none is widely used as a standard technique in diagnostic laboratories in Australia or New Zealand.

Some recent reviews have highlighted the potential but limited application of such techniques at the present time.^{16,17}

ELISA for Nematode Infections

Early studies using crude whole worm extracts of nematodes were confounded by cross-reactions between the closely related trichostrongyle species. Attempts to develop more specific tests have relied on specific recombinant proteins. The studies have tended to be restricted to monitoring nematode infections, especially *Cooperia* and *Ostertagia* infections in cattle in Europe, on a herd basis rather than for the diagnosis of individual animal infections. A commercial ELISA for herd health monitoring of *O ostertagi* antibodies in milk has been developed (SVANOVIR® *O. ostertagi*-Ab ELISA, Svanova Biotech AB, Uppsala, Sweden; see <http://www.svanova.com>), but has not been validated in Australia.

Faecal Antigen Detection (FAD) Methods

Three capture ELISA-based tests for the detection of *H contortus*, *Trichostrongylus* and *Teladorsagia (Ostertagia)*, respectively, infections in faeces of infected sheep have been developed in Australia, and evaluated in pen studies with sheep either monospecifically dosed with larvae, single combination infections or repeated small trickle doses.⁴⁷ All three tests could detect both mature and immature infections, although the *Trichostrongylus* and *Teladorsagia (Ostertagia)* tests were more effective with adult or late stage immature infections. The FAD

method performed as well as or better than faecal egg counts in defining burdens and could identify individual species in mixed infections.⁴⁷ At present these tests have not been adopted for monitoring natural infections.

PCR for Nematode Infections

PCR techniques have been used for identification and diagnostic purposes for trichostrongyle nematodes and other parasites, as well as to differentiate strains or isolates within species.^{17,48} Depending on the purpose of the test, a range of PCR procedures have been used including conventional PCR, targeted against various regions especially the first or second internal transcribed spacer (ITS-1) and (ITS-2) of nuclear ribosomal DNA. Current work has shown considerable promise for the development of real-time PCR for the diagnosis of nematode infections in sheep to genus or species level.¹⁷ The assays would not be field tests, but would probably be used as an extension to FECs and replace larval cultures. More recently real time (RT)-PCR has been applied for the molecular diagnosis of anthelmintic resistance notably BZ-resistance and ML (ivermectin)-resistance.^{49,50} The feasibility of RT-PCR for the differentiation of species of infective L3 from intestinal nematodes of naturally infected sheep has been described⁵¹, and attempts to apply such techniques to larvae recovered from pasture with limited success has been reported.⁵²

Accreditation and Proficiency Schemes

In Australia, laboratories may be accredited under a number of agencies, the most common being the National Association of Testing Authorities, Australia (NATA). Veterinary diagnostic laboratories are either accredited under ISO/IEC 17025:2005 for veterinary testing, or certified for quality management under ISO 9001: 2001. NATA-accredited laboratories are required to participate in Proficiency Testing (PT) schemes where these are available. Two such parasitology PT schemes were operating Australia. The first is for faecal nematode egg counts (FECs) only and is operated by the Albany laboratory of the Department of Agriculture and Food, WA (DAFWA). The second, which covered FECs, liver fluke egg counts and nematode larval differentiations, was organised by Elizabeth Macarthur Agricultural Institute of the NSW Department of Primary Industries, but has recently been discontinued.

Anthelmintic Efficacy Testing

Guidelines for standardised methods for evaluating the efficacy of anthelmintics have been developed to help resolve some of the inconsistencies of regulations in different jurisdictions around the world. Although complete harmonisation has not yet been achieved major advances have been made.

WAAVP Guidelines

The World Association for the Advancement of Veterinary Parasitology (WAAVP) was the first organisation to have developed a series of anthelmintic guidelines for all host species. The guidelines, which relate to ruminants, have recently been revised as a second edition by Wood et al.⁵³

APVMA/VICH Guidelines

The Australian Pesticides and Veterinary Medicine Authority (APVMA) (formerly the National Registration Authority for Agricultural and Veterinary Chemicals, NRA) has generally accepted the WAAVP guidelines.

The *International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH)* is an international programme of co-operation between

regulatory authorities and the animal health industries of the European Union, Japan and the USA. Australia and New Zealand have observer status on this but are not signatories to the VICH. The VICH guidelines for anthelmintic efficacy VICH GL7 (general requirements), VICH GL12 (bovines), VICH GL13 (ovines) and VICH GL14 (caprines) have been summarised⁵⁴ as have those for equines, porcines, canines, felines and poultry.⁵⁵

The current Australian guidelines consist of Veterinary Guidelines no. 51 Guidelines for the efficacy of anthelmintics — General requirements; v52 specific recommendations for bovines; v53 specific recommendations for ovines; v54 specific recommendations for caprines. They have been adopted in whole from the VICH guides of the same name.

However, efficacy standards in Australia are higher than those suggested in the VICH because of Australia's unique environmental/geographic conditions, parasite burdens and population dynamics with claims for treatment/control being efficacies >95% for gastrointestinal helminths and lungworms, while claims for persistent effectiveness are >99% (sheep/goats) and >95% cattle. Claims for treatment for liver fluke are >90% efficacy. The claim for persistent effectiveness is due to the prominence of *H contortus* in Australia. APVMA also requires that most of the trials to confirm efficacy in the field be conducted within Australia under typical farm management practices in relevant regions.

A second variation is that APVMA does not believe that with respect to statistics in Section 4.1 *Geometric versus arithmetic means* of the Guideline V51 that the adoption of geometric means where data are non-normally distributed should be the sole means of interpreting trial data. Where the arithmetic mean shows marked variance from the geometric mean then the arithmetic mean will be taken into consideration.

Additional guidelines for the registration of combination anthelmintics are contained in a separate NRA Guideline No. 26 (1996) however, these were produced when only resistance to benzimidazole (BZ) and imidothiazole (levamisole, LEV) and BZ/LEV combinations were available and do not take into account the recent development of triple and quadruple combinations.

Full details of APVMA's Manual of Requirements and Guidelines for Veterinary Applications (Vet MORAG, ver 4.1, 2007) are available from the APVMA website at http://www.apvma.gov.au/MORAG_vet/MORAG_vet_home.shtml.

Diagnosis of Anthelmintic Resistance (AR)

When the earlier versions of this document were published, anthelmintic resistance in nematodes of sheep was confined mainly to the benzimidazole (BZ) or 'white' drenches; imidothiazole (LEV: levamisole/morantel 'clear' drench group) or BZ/LEV combinations. Resistance in nematodes of cattle was largely undocumented. Since then worms resistant to avermectin/milbemycin (AM), also referred to as macrocyclic lactones (ML), have become relatively common in certain regions of Australia and New Zealand. Increasing the spread of ML-resistance in sheep is believed to be due at least in part to selecting resistant populations by treatment at times when there are few worms in refugia (those stages such as eggs or larvae on pasture that are not accessible to anthelmintics). Methods to control the spread of ML-resistance have been suggested by an expert panel.⁵⁶

The current status and the mechanisms and possible means of control of anthelmintic resistance have been reviewed.^{57,58,59}

A WAAVP paper on methods for the detection of anthelmintic resistance was published

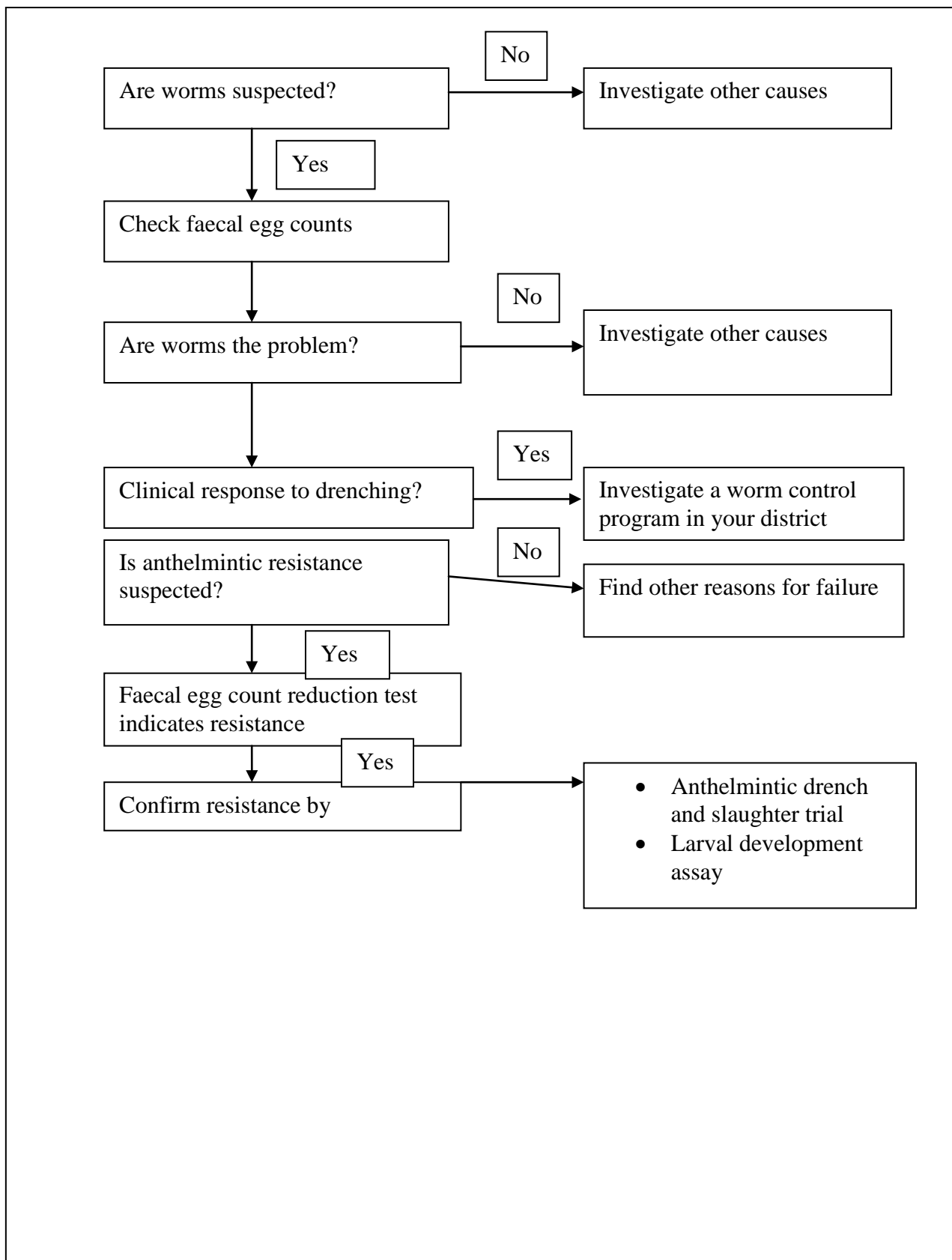
before the current widespread nature of AR was established.⁶⁰ The authors of that paper have recently reviewed the topic.¹⁴ They conclude that at this time there is a great need for improved methods especially for ML-resistance and for use in cattle, which need to be validated and tested in laboratories in different parts of the world before WAAVP could make recommendations.

Suspicious of drench resistance are often based on a vague feeling by the farmer or veterinarian of failure of the anthelmintic treatment, which could be due to a number of factors unrelated to actual drench resistance (for example, inaccurate estimates of animal weight, failures of drenching equipment, use of outdated chemical, poor drenching technique, 'missed' animals etc.).

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 steps to be taken to clarify the situation (Figure 4).

Figure 4: Investigation of a suspected anthelmintic failure



Worm Infections and Anthelmintic Efficacy Diagnosed on Faecal Egg Counts

Faecal egg counts are the simplest method of determining worm infections. If a worm infection is suspected, faeces for egg counting and cultures should be taken. If egg counts indicate that drenching is required, then drench and 10-14 days later take faecal samples to check anthelmintic efficacy. A shorter interval after drenching of 7-10 days has been recommended for New Zealand conditions.⁶¹

To test for drench efficacy the chosen anthelmintic is used at the manufacturer's recommended dose rate (RDR), whereas for detailed resistance testing the discriminating dose for the particular worm genus, which may be less than the RDR, should be used. For example, for resistance testing with *H contortus*, use closantel at one-third dose rate as this removes any residual effect, while half dose rate (100 µg/kg) of ivermectin (IVM) to detect emerging ML resistance has been suggested.⁶²

At drenching, take individual faecal samples from 10-15 animals in a mob for egg counts and cultures (see earlier sections on egg counting techniques and faecal cultures).

Drench sheep with the anthelmintic (see Appendix 3) at the RDR

Take the following precautions:

- calibrate the drench gun,
- drench to the top weight of the mob (if large differences in weigh draft into several size ranges and weigh or estimate weight of heaviest),
- use clean equipment, and
- ensure that the animal swallows the drench.

Take further faecal samples from these sheep 10-14 days after drenching (7-10 days in New Zealand) for egg counts 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 (epg) average for the group is sufficient to proceed with the Faecal Egg Count Reduction Test (FECRT see following section). Some laboratories may use a lower limit such as 200 epg)

The grazing animal normally carries a mixed parasite infection. The genera that compose the population are 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. If *Haemonchus* makes up a significant proportion, then narrow spectrum drenches may be applicable such as naphthalophos, closantel or pyraclofos. Even when levamisole resistance is present in *Trichostrongylus* and *Teladorsagia* it may still be effective against *Haemonchus*.

For resistance testing FECRTs without larval differentiations are not acceptable. In order to determine whether resistance is a problem in other genera, it may be necessary before conducting a FECRT to remove any *Haemonchus* unless resistance to *Haemonchus* is not an issue (as in WA) or they comprise only a minority of the population.

Egg counts reflect the presence of mature egg-laying worms in the gastrointestinal tract. If faecal samples for egg counting and culture are taken 10-14 days after 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-infection. Incoming larvae from the paddock will not be mature by day 14 (except possibly *H contortus*, or *Cooperia oncophora* in cattle) and their presence will not be reflected in the egg count.

Some drenches, for example, benzimidazoles (and also MLs) suppress egg laying by adult worms that survive the drench for up to five days. 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 programmes advocate routine faecal egg count monitoring of flocks before and after drenching. If drenches are omitted from a programme then regular monitoring becomes essential.

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:

- Choice of Anthelmintic
 - Was the correct drench family used? An understanding of their action class is essential for appropriate anthelmintic choice, for example, a broad-spectrum anthelmintic, such as levamisole (LEV), which may still be effective against *Haemonchus* may no longer control *Trichostrongylus* or *Teladorsagia* (*Ostertagia*).
- Administration of Anthelmintic
 - Did the animal 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 animal swallow the drench?
- Parasite Factors
 - Response to drenching can be masked by the following:
 - 1) Larvae unaffected by treatment will continue to develop rapidly and produce signs of disease.
 - 2) Rapid re-infection from the pasture.
 - A closantel drench will stop development of incoming *Haemonchus* larvae. Broad-spectrum drenches have no effect on incoming larvae (except for sustained release capsules and moxidectin, especially the recently introduced long-acting injectable versions).
 - A heavy burden of *H. contortus* in weaners will cause severe anaemia and mask benefits of the drench even after removal of worms because depletion of iron reserves will cause the weaners to take longer to recover.
- Concurrent Conditions
 - Other factors that can hinder recovery are:
 - Illthrift due to poor nutrition; and
 - Bacterial or protozoan diseases, for example, coccidiosis, salmonellosis, eperythrozoonosis (mycoplasmosis)

Mycoplasma ovis (formerly *Eperythrozoon ovis*) is a blood protozoan, which can cause anaemia in weaner sheep and reduce tolerance to mustering. Most outbreaks are observed during late winter and spring in high rainfall areas. Mosquitoes or flies on wounds may transmit the parasite mechanically.⁶³ Affected sheep should be handled carefully, given nutritious feed and a sufficient water supply, sheltered from the elements and disturbed as little as possible.

Resistance Diagnosed on Faecal Egg Count Reduction Test (FECRT)

The FECRT answers the question: Will this drench kill all the worms in my sheep?

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 (Note: strict annual rotation of drench groups is no longer recommended practice⁶⁴) on individual properties.

This test should be performed when adopting or modifying a strategic worm control programme.

Alternatively, if the adopted worm control programme appears not to be effective, then the FECRT is recommended to reassess anthelmintic resistance on the property in question.

There are two aspects to this procedure: the field trial (see below) and the laboratory procedures.

Field Trials

Planning

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

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. The same sheep, preferably 15, need to be sampled both at and after drenching in *H contortus*-endemic zones, as egg counts can vary considerably between sheep.

Drenching history of test sheep

Sheep should not have been drenched with a broad-spectrum anthelmintic in the last four weeks and preferably the last 10 weeks, or with closantel in the last 10 weeks, or with persistent MLs including long-acting moxidectin, and capsules. Undrenched sheep are preferred.

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 programmes require 500 epg.

Anthelmintics to be tested

(See Appendix 3; Select from BZ; levamisole; BZ/levamisole combination; ivermectin;

naphthalophos; closantel and/or capsule (Also moxidectin/abamectin and triple or quadruple combination, for example, Triton, Q-drench, Hatrick). For each property, all appropriate anthelmintics should be tested.

Mixtures and x1.5 or x2 doses may be appropriate, if the resistance status at manufacturer's recommended dose rate (RDR) is known and if it is quite close to the cut-off level (95%).

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.

Dose rates to be tested

Half-dose ivermectin has been used for identifying emerging resistance against MLs.⁶² At the one-third dose, the residual activity of closantel, which normally protects against incoming larvae, is eliminated. The efficacy of the anthelmintic against the host worm burden can then be assessed.

Time of the Year

Tests performed soon after a summer drought (or in WA where sheep may be drenched before moved on to crop stubble) and/or refugia may overestimate the degree of resistance on the property. The populations of worms in the host at that time are the resistant survivors of the last drench. No larval pick-up from the paddock, due to the drought, would have occurred to reveal the true situation for that property.

Equipment

Scales to weigh the sheep (bathroom scales are adequate).

Colour marking paint (for example, SIROMARK or easily scorable dry raddle, or colour-coded ear tags, to indicate drench groups of sheep.

Faecal collection bottles (jars) or plastic bags.

A permanent marking pen for labelling collection containers.

Drench gun(s) and backpack.

Associated paper work, that is, Laboratory Advice Sheets/Key lists/pre-printed labels, etc.

Operation

On Day 1 of the Test

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) is optimal but depends on the number of drenches to be tested. The sheep should be this year's lambs, of even size and preferably not previously drenched.

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.

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 groups, No. 2 to the second and so on. The common practice of allocating the first 10 sheep into the first group, second 10 to the second groups and so on does *not* constitute proper randomisation due to hierarchical groupings. Colour mark the sheep sequentially down the race using head, neck, back, rump marks until all sheep are accounted for. There should be 15 sheep per group.

Collection of at-treatment samples

Some laboratories prefer to collect faecal samples from all animals (including controls) before and after treatment. This depends on the method required to calculate FECR.^{66,67,68,69,70} It is more common when weaners are not available, where group means are low (200 epg or less), where it is expected that the egg counts in samples from the control group will have increased substantially in the period before and after treatment, such as with *H contortus*.

Drench

Dose sheep at the required dose rates. Always check the label to confirm that the dose is that recommended by the manufacturer. This is especially important when using concentrated (often referred to as low volume, LV or reduced volume, RV) drenches. Use of higher than recommended dose rates, for example 1.5 or 2 times may be justified if it is already known that resistance is present at recommended dose (1x) rate. Use of closantel at one-third recommended dose rate (removes residual activity)⁷¹, and ivermectin at one-half recommended dose rate (100 µg/kg) have been used to detect 'emerging' resistance.⁶²

Hints

If using the same drench guns for different drenches, use the clear drenches before the white drenches to prevent possible blockages.

Rinse and clean drench guns and packs with water between each test drench.

Calibrate drench guns before using each test drench, that is, squirt 10 doses into a measuring cylinder.

On Day 2 of the Test

The second day of the test is 10-14 days after the 'test' drenching (Note: in recent trials in New Zealand on the prevalence of resistance, samples were collected only 7-10 days after treatment^{72,73}, which may not be sufficient time to allow temporary suppression of worm egg laying to have diminished. However, as 92% of farms were found to be carrying anthelmintic-resistant *Cooperia*, this suggests that temporary suppression of FEC was not of any real moment.)

Collect faecal samples

Collect rectal 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 the laboratory submission form.

Submit faecal samples to the laboratory. Faecal samples and the completed submission form should be despatched immediately to the laboratory.

Where transport will take longer than overnight, despatch samples in an insulated container containing a freezer brick wrapped in several layers of newspaper. NB. Storage of samples for prolonged periods below 4°C will prevent *H contortus* eggs from hatching. Samples should be stored at 10°C. Differences in the abilities of eggs of various worm genera to survive refrigeration can, in some cases following cold storage for as little as 24 hours, lead to significant changes in the percentage composition of their third stage larvae.⁷⁴

*Laboratory Procedures**Processing Samples*

Some laboratories prefer to use one of the bulk egg counting techniques (see earlier section) to process faecal samples into drench groups. However, it is preferred to use individual counts.

Prepare one bulk larval culture per drench group.

Read larval cultures seven to eight days later. See Figure 1 for larval differentiation.

Calculation of Results

The recommended procedure for the conduct of a FECRT is published.⁷⁵ The worked example is adapted from that information (see Table 5). Two computer programs have been developed by CSIRO to assist with statistical calculations and interpretation of FECRTs.⁷⁶ The original 'RESO' programs were either DOS or Lotus 1-2-3 programs, which are now obsolete. A Microsoft Excel spreadsheet version is available from the *Sustainable control of internal parasites of sheep* (SCIPS) web site at the University of Sydney (<http://www.vetsci.usyd.edu.au/sheepwormcontrol/>)

Resistance is said to exist if the reduction in the arithmetic mean from the drench groups is less than 95% (when compared with those from untreated sheep at day 10-14 or the treated group prior to treatment), and in which the lower 95% confidence limit is less than 90% reduction level. (Note: Calculations using geometric means of log-transformed counts are sometimes used for calculation of percent efficacy but should not be used for the estimation of resistance). In New Zealand it was found that if the mean FECRs were less than 95%, the lower confidence interval was always less than 90%. These findings suggest that little practical purpose is served by further consideration of the lower confidence limit.⁷⁷

Worked example: Counts 10-14 days after treatment (Table 5)

**Table 5: Faecal egg count reduction test — worked example
(counts after treatment only, 10 days)⁷⁵**

Group no. (i)	Faecal egg count in epg		
	Control	Anthelmintic A	Anthelmintic B
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 ¹	10	10	10

¹ Total number of sheep in the group = n_i : ($N = \sum n_i$)

	Control	A	B
Arithmetic mean count $\bar{X}_i = \sum_j X_{ij}/n_i$	443	9	210
Variance of counts $s^2_i = [\sum_j X_{ij}^2 - (\sum_j X_{ij})^2/n_i]/(n_i-1)$	74062	260	20300
Percent reduction $R = 100(1 - \bar{X}_t/\bar{X}_c)$	0	98	53
Variance of reduction on log scale $V = [s^2_t/n_t \bar{X}_t^{-2}] + [s^2_c/n_c \bar{X}_c^{-2}]$		0.36	0.08
Approximate 95% confidence interval for R $100[1 - \bar{X}_t/\bar{X}_c] \exp(\pm 2.1\sqrt{V})$			
Upper confidence limit $100[1 - (\bar{X}_t/\bar{X}_c) \exp(-2.1\sqrt{V})]$		99	74
Lower confidence limit $100[1 - (\bar{X}_t/\bar{X}_c) \exp(+2.1\sqrt{V})]$		93	13

Where i denotes either the treated (t) or control (c) groups,

j denotes each sheep in the group,

s^2_i denotes the variance on the arithmetic scale,

calculated as above or:

$$s^2_i = \sum_j (X_{ij} - \bar{X}_i)^2 / (n_i - 1)$$

Other Statistical Methods for Calculating Resistance

Earlier reviews of both the methodology and calculations were provided by Presidente⁶⁸, and Sangster and Dobson.⁵⁸ Recently, Torgerson et al compared a number of mathematical techniques and found that the maximum likelihood technique with a negative binominal distribution would aid in the detection of AR at an early stage.⁶⁹ The WormBuster programme in Queensland uses a spreadsheet, Q_FECRT, based on arithmetic means of treatment and control groups at and after drenching for worm burdens that follow a negative binominal distribution in older, more immune sheep. Computer simulation studies showed geometric means did not produce satisfactory results.⁷⁰

McKenna has analysed an historical series of 210 FECRT studies to determine which of the various methods using combinations of samples both before and after treatment of control and treated groups is the most accurate in detecting AR.^{66,67} While the most complex of the calculation methods (sometimes referred to as the Presidente method of pre- and post-sampling of all groups), McKenna's FECRT₁⁶⁶ is the most technically correct. He concluded that there were no significant differences in the number of populations that qualify as resistant (that is, FECRT <95%). Sensitivity and specificity of the different methods of calculating FECRT were compared in 61 of the 210 studies where both egg counts and controlled slaughter trials were conducted.⁶⁷ Although FECRT₃ and FECRT₄ were slightly more sensitive (96%), compared with 92% for FECRT₁ and FECRT₂, there was no statistically significant difference and specificity was 100% in all cases. McKenna⁶⁷ concluded that no performance improvements are likely to be gained by the use of a more complex FECRT than a simpler one, and that the cost savings from reduced laboratory expenses would be considerable.

Further, support for using a composite faecal egg count for testing drench resistance was proposed.⁷⁸ A criticism of composite samples is that it is not possible to differentiate valid cases of resistance from cases where one or two animals per group have been missed during drenching. To clarify this McKenna⁷⁸ analysed a total of 373 FECRTs with individual FECs involving 10 or more animals per group, and a mean strongylid pre-treatment count of 150 epg, and recorded the number of positive post-treatment FECs. Of 88 cases with one or two sheep with positive post-treatment counts only 10 were classified resistant (<95% reduction in FECs). Of these 10 cases, only two were clearly related to sheep being missed. The caveat for the use of composite samples is that individual faecal samples should still be collected and that pooling and thorough mixing of equal weight of faeces from each sheep in the group is required.⁷⁹ Better still, faecal samples for composite counting should be pooled in groups of 5. Where egg counts within a group that is treated are not consistent, samples from individual sheep within the group with the highest count should be recounted individually until the sample representing the sheep that missed the drench is identified and eliminated from the calculations.²⁷

An alternative method is to use an individual animal FECRT⁷⁹, which gives equal weight to every tested host. Individual FECRT produces lower values than average-based FECRT in most cases but provides a reliable evaluation when egg counts are above 300 epg and at least 10 animals are tested.

The repeatability of FECRTs within animals on farms with confirmed anthelmintic resistance when large numbers of animals are tested was examined by Miller et al.⁸¹ They calculated undifferentiated (that is, no larval cultures conducted) FECRTs using FECRT1, FECRT2 and FECRT3 equations using arithmetic means. They also calculated efficacy against specific genera. Calculated efficacies differed between equations and studies that did not incorporate an untreated control yielded significantly lower estimates of efficacy. Faecal cultures varied

widely but did not differ between high, medium or low FEC groups, except where *Haemonchus* was more common and *Cooperia* less common in high-FEC samples.⁸⁰ They concluded that caution was needed in interpreting FECRTs especially in the 90-95% reduction range close to the cut-off for declaring resistance.

Diagnosis of Resistance using Anthelmintic Drench and Slaughter Trials

The in vivo drench and slaughter trials, because of their magnitude and cost are usually investigational or research tests only.⁵³

Field trials without the use of worm-free host lambs can be considered as a means of confirming resistance on a property using, say, 20 naturally infected weaners in an on-farm FECRT and slaughtering 5 or 6 animals with the highest FEC 10-14 days after treatment from treatment and control groups. Ideally animals should be kept off pasture during such trials, or if on pasture the recently acquired immature stages should not be included in the calculations.

More comprehensive studies using experimental infections with the new 'resistant isolate' will be needed to fully characterise the new resistant worm strains.

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 parasites to the test anthelmintic is compared with the response of known susceptible and known resistant larvae for that particular anthelmintic. Anthelmintics are usually tested at different dose rates to construct a dose/response curve, which may be transformed to produce a straight line.⁵⁸ Results are compared statistically to determine whether changes in the angle and position of response lines are significant.

Worm-free Host Lambs

Host lambs should be less than seven months old, reared on concrete, wire or wooden slats and fed a prepared ration. Alternatively, purchased lambs of suitable age and carrying low worm burdens can be drenched with a short acting ML (such as ivermectin or abamectin) at twice the manufacturer's recommended dose on two occasions, one week apart. If there are suspicions that a resistant population is present it may be necessary to use a different 'quarantine' drench, which could be a triple or quadruple combination.

Egg counts by the McMaster Method (see earlier section) must be zero before the start of the trial. After the second dose of 'clean out' drench, wait at least seven days (longer with some drenches such as abamectin and with *Haemonchus*) before infecting with the suspect resistant larvae.

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.

Production of Infective Larvae

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 trials are about:

2500-5000	<i>Haemonchus contortus</i>
2000-3000	<i>Teladorsagia (Ostertagia) circumcincta</i>
3000-6000	<i>Trichostrongylus</i> spp.

per sheep based on WAAVP guidelines.⁵³ The proportion of the differing species should be determined from a larval differentiation of species from mixed field infections.

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

Infection of Worm-free lambs

Infect the lambs with the required number of infective larvae suspended in water either by intra-ruminal injection or per os using a dosing needle.

Determine egg counts of infected lambs between days 23 and 27.

Weigh each animal.

Allocate lambs into groups by stratified randomisation according to body weight and faecal strongyle egg counts.

Testing the Anthelmintic(s)

On days 24-28 after infection, treat all animals with the appropriate 'test' anthelmintic or anthelmintics if multiple resistance is suspected. A second anthelmintic, from a different chemical class, should be tested on another set of sheep. (This could be from a different drench family or it could be another member of the MLs (moxidectin/abamectin/IVM) if resistance against a particular ML is anticipated). As with FECRT, it may be appropriate to have a group treated with a reduced dose rate to determine whether an 'emerging' resistance is being detected (for example, using one-third dose of closantel, or one-half dose of ivermectin). Traditionally⁸¹ testing several dose rates, such as 0.5, 1.0, and 2 times the recommended dose, have been used as logarithm-transformed dose rates are used in analysis.⁵⁸ A reference group of sheep infected with a strain of the particular nematode species susceptible to the anthelmintic being tested is also recommended. Increasingly the availability of populations of multiple species of nematodes with resistance to multiple drug families is making such treatment and slaughter trials complex and expensive.

Slaughter all animals 7-10 days after treatment. Collect the appropriate portions of the gastrointestinal tract at necropsy. Process each section separately (see section on total worm count).

Calculation of Results

Calculate the percentage efficacy. For each anthelmintic, compare the average number of worms per dose rate with those of the control group using arithmetic or logarithm-transformed geometric counts

Produce dose response lines.^{41, 58}

Compare these lines of dose response with those produced for the same anthelmintics against susceptible strains of the same nematode species.

Definition of Resistance

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 is similar to the resistant strain (See Sangster and Dobson⁵⁸ for explanation).

Diagnosis of Resistance to Broad-Spectrum Anthelmintics by *in vitro* Larval Development Assay

The Larval Development Assay (LDA) is an *in vitro* technique for detection of resistance to broad-spectrum anthelmintics 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 resistance to benzimidazole (BZ), levamisole/morantel (LEV), BZ/LEV combinations and ivermectin (ML). Nematode eggs are isolated from a *bulk* faecal sample, applied to the wells and allowed to develop to infective L3 larvae over six to seven days. Eggs in wells will hatch and develop through L1 and L2 stages depending on the concentration of the anthelmintics. Thus, isolates resistant to an anthelmintic will develop in wells containing higher concentrations than susceptible isolates.⁸²

The LDA offers the following advantages over existing techniques:

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

A variation of the larval development assay is an *in vitro* larval migration assay (LMA) using the ability of resistant L3 (usually *H. contortus*) cultured in the presence of varying concentrations of drug to migrate through micropore sieves or filters.⁸³ This method in 96-well plate format has been used to detect closantel resistance^{83,71,84} and has been applied to resistance surveys.⁸⁵ Some modifications incorporating migration through agar have also been developed to detect ML resistance in *Haemonchus* but were not suitable for *Trichostrongylus* or *Teladorsagia* (*Ostertagia*).⁸⁶

Limitations of the Test

The test is unable to use combinations other than the 1:1 BZ/LEV and is unable to detect resistance to the narrow spectrum organophosphate naphthalophos (NAP) or closantel. It is of limited usefulness because of the unreliability of ML results (refer to Palmer et al⁶²) particularly for ivermectin resistance in *Teladorsagia* (*Ostertagia*). Attempts to use other ML analogs to improve the effectiveness of the test have led to only limited success.⁸⁶

Commercialised Tests (for example, Drenchrite™)

The LDA was developed into a commercial test by CSIRO and licensed as Drenchrite™ to Horizon Technologies (license subsequently transferred to Bioniche). Since 2002 the only commercial laboratory in Australia that offered this test (irregularly) was the NSW DPI Parasitology Laboratory at Elizabeth Macarthur Agricultural Institute (EMAI), but this has been discontinued (January 2008).

Field Collection

Randomly select at least 10 sheep from the flock to be tested and collect no less than 100 g of faeces as pool sample.

Take representative egg count on the bulked sample. Submit samples with an epg >100 for LDA (200 epg is EMAI cut-off).

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 ambient temperature. The time between collection and assay should be less than seven days.

Laboratory procedure

Eggs are separated by a modified sucrose flotation technique. (Drenchrite™ uses filtration and then differential centrifugation)

Eggs are applied to pre-prepared plate and incubated at 22°C for 7 days by which time control (no drug) wells have developed to infective L3 larvae.

Larvae are killed with iodine and development assessed by two methods:

- 1) Qualitative (by eye). Transition between L3 (uninhibited) and L1-2 (inhibited) wells can be assessed by eye to identify well number at which about 50% inhibition of development occurs.
- 2) Quantitative (counting). Proportions of L 1-2 and L3 (and eggs) can be counted and data computer fitted by logit-log concentration model to derive LD₅₀. In the commercial kit a table is provided which relates the counts to percentage efficacy.

Calculation of Results and Interpretation

Approximate LD₅₀ values for field isolates can be obtained by conversion from well number using the concentration factor **b**, where **b** is the concentration in well No.1, thus

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

where **a** is well number in which inhibited L1-L2 larvae and uninhibited L3 are in roughly equal abundance (*that is the "critical well" in Drenchrite™*).

NB. Qualitative LD₅₀s normally agree within two-fold of the computed quantitative value.

Resistance factors (*RFs*) can be calculated by:

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

Where **a** and **c** are the well numbers in which approximate LD₅₀ values of field and susceptible isolates are noted.

Alternatively, if a susceptible isolate is not run with the field-isolate (Note: Drenchrite™ no longer run a susceptible isolate as control), *RF* can be calculated as:

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

where SLD₅₀ is the data base susceptible value and **b** is concentration

The *RF* for a field isolate is used to predict the faecal egg reduction achievable for the field isolate from 40 sets of field FECRT and LDA data.

Correlation of LD₅₀ with In Vivo Efficacy

Prior to the commercial release of the DrenchRite™, studies were conducted where comparative performances of the LDA with FECRT as an *in vivo* measure of anthelmintic efficacy were assessed. A table in the DrenchRite SOP lists the critical well (LD₅₀) at which half larvae developed with an equivalent efficacy of BZ, LEV and Combo for each predominant species with upper and lower 95% confidence intervals.

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Hall CA. *Diagnosis of Anthelmintic Resistance in Sheep and Goats*. Australian Bureau of Animal Health, Canberra. 1982.

Hall CA *Australian Standard Diagnostic Techniques for Animal Diseases, No. 46, Diagnosis of Anthelmintic Resistance in Sheep and Goats*. CSIRO for the Australian Agricultural Council, East Melbourne. 1987.

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APPENDICES

Appendix 1: Preparation and use of Reagents

Caution: check appropriate Occupational Health and Safety issues before use.

Embalming fluid

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

EF is a 20% aqueous solution of ethanol (C₂H₅OH) with a little formaldehyde, Lysol[®] (see below) and glycerol (CH₂OH.CHOH.CH₂OH) added. It will preserve small ruminant gastrointestinal tracts for at least two weeks. Formalin by itself is unsuitable for this purpose as it makes the tract stiff and worm recovery very difficult.

To make 20 L of EF, mix:

Formalin (37% formaldehyde)	400 mL
Lysol [®]	400 mL
Glycerol/glycerine	1200 mL
95% ethanol (absolute alcohol)	4 L
Tap water	14 L

Shake before use.

Removal and preservation of small ruminant gastrointestinal tracts for total worm counts.

Open carcase and locate various organs of the gastrointestinal tract.

Tie (with string) the abomasum at the 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. Tie off each section of the bowel with a double tie

Inject 20 mL EF into each of the abomasum, small intestine, caecum and colon. Knead organs to mix EF throughout contents.

Place each of abomasum, small intestine, caecum and colon into separate heavy duty plastic bags (450 x 300 mm, good for small sheep) and add 300 mL EF. Swirl fluid to ensure good contact with tract. Pour off excess preservative. Seal each bag by tying a knot, excluding as much air as possible. Place each bag in a second bag for extra security against spillage and seal similarly.

Preserved tracts held at ambient temperature will be suitable for total worm counts for at least two weeks. Transport in an insulated, rigid container.

Lactophenol

Lactic acid, CH ₃ CHOHCOOH	1 part
Phenol, C ₆ H ₅ OH	1 part
Glycerol/glycerine	1 part
Distilled (or reverse osmosis, RO) water	1 part

Mix ingredients

Place specimen to be cleared on microscope slide, cover with lactophenol and warm gently until fumes begin to rise (Caution: Fumes are toxic, thus such procedures should only be conducted in a fume hood)

Add more lactophenol if necessary, coverslip and examine

NB. Lactophenol will cause some shrinkage.

Flotation Solutions

Sodium chloride, saturated solution specific gravity of 1.20

Dissolve commercial grade sodium chloride (superfine kiln dried or pool salt) 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 through cheese cloth or muslin to remove the debris.

Measure the specific gravity with hygrometer or weigh; 100 mL should weigh 120 g.

Magnesium sulphate, saturated solution specific gravity 1.3

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

Parasitological iodine

Iodine, I ₂	30 g
Potassium iodide, KI	40 g
Water to	100 mL

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

Storage Solution for Helminths

Alcohol	
70% alcohol (ethanol)	95 parts
Glycerine/glycerol	5 parts

Formalin, 5% solution

Commercial formalin (37% formaldehyde)	5 parts
Water	95 parts

Buffered Neutral Formalin (BNF) 20 L

Disodium hydrogen phosphate, anhydrous Na ₂ HPO ₄	148 g
Sodium dihydrogen phosphate, dihydrate NaH ₂ PO ₄ .2H ₂ O	101 g
Formalin (40%)	2 L
Water to make	20 L

Dissolve phosphates in hot water, cool then add formalin and make up with water.

Helminth Storage Solution (NSW DPI recipe)

95% Ethanol (absolute)	70 parts
Glycerol	5 parts
Formalin (37% formaldehyde)	3 parts
Water	22 parts

Lysol[®]

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

Vermiculite

Vermiculite is hydrated laminar magnesium-aluminium-iron silicate, which resembles mica in appearance. When subjected to heat it expands (“exfoliates”) into worm-like pieces. It is used in horticulture as an inert potting mixture for hydroponics. It has a very high water retention capacity.

Pepsin — Hydrochloric Acid Digestion Liquid

Pepsin is available from various laboratory suppliers. The potency of different batches varies: use 10 g of 3 000 units/L or 2 g of 150 000 units/L..

Add concentrated hydrochloric acid (30 mL) to 1 L water.

Stir to dissolve pepsin.

One litre of *Hydrochloric Acid Digestion Liquid* is sufficient to digest about 500 g of tissue.

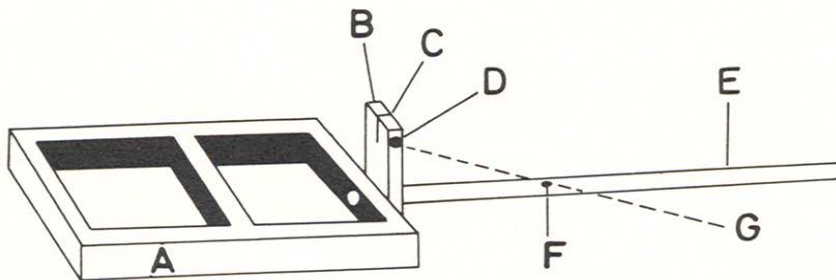
Appendix 2: Equipment

Gut Runner

For necropsy of intestines (source: Skerman and Hillard 1966)⁴².

See Figure 5 and following legend)

k. The "gut-runner".



k. The gut-runner with blade in position.

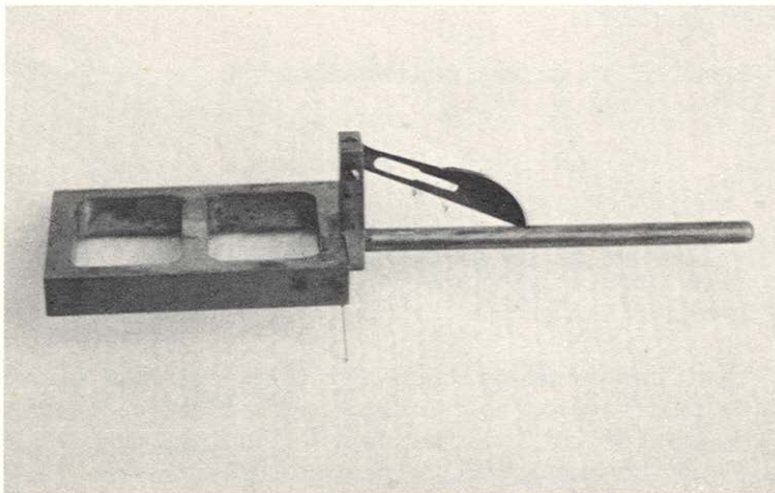


Figure 5 (a) the 'gut runner' and (b) the 'gut runner' with blade attached

Material: brass or other non-corrosive material.

Letters next to text refer to labels on Figure 5a

- A. 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.
- B. Blade carrier centrally placed at one end of base framework. Overall height 4 cm (top to bottom of brass framework). Width 1 cm. Thickness 8 mm.
The base framework and blade carrier can be assembled from separate pieces, or cast as one piece.
- C. Blade groove cut centrally into the top of blade carrier 1.5 cm deep, 0.5 mm wide.
- D. 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.
- E. Guide rod, a solid cylindrical brass rod, diameter 7 mm, length 12 cm, one end embedded and welded into the brass framework, passing centrally through the blade carrier.
- F. Blade-tip anchor hole. This small hole of 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 22A). It is drilled at a suitable angle to receive the tip of the blade.
- G. Angle of inclination for blade-tip anchor hole, taken from the bottom of the blade groove through the anchor hole position.

Whitlock Counting Chambers

A variety of universal and McMaster counting chambers are available from:

JA Whitlock & Co, PO Box 51, Eastwood NSW 2122, phone 61 2 9638 1142

http://www.whitlock.com.au/slides/JAWCO_Home.htmh. Accessed 29 April 2008.

Endecott Sieves

A range of Endecott sieves, and accessories can be obtained from Crown Scientific (www.crownsscientific.com.au. Accessed 28 April 2008).

Approximate conversions (various sources)

Mesh (Strands per inch)	Metric system (μm)
16	1000
30	600
35	500
40	400
50	300
70	200
80	180
100	150
150	100
200	75
300	50
400	38

Appendix 3: Anthelmintics for the Control of Gastrointestinal Nematodes in Sheep

The most comprehensive listing of registered anthelmintics is available through the PUBCRIS database of APVMA. Another more accessible listing is available from the NSW DPI website, see Love and Cook (2006) Primefacts 152: Registered drenches for sheep worms (see Table 1). Lists of sheep anthelmintics are also provided in INFOPEST (QDPI&F), and at the WormBoss web site (www.wormboss.com.au).

Appendix 4: Anthelmintics for the Control of Gastrointestinal Nematodes in Goats

Only a very small number of anthelmintics are registered for use in goats; see Table 1 from Love and Cook (2006) Primefacts 152: Registered drenches for sheep. Available at <http://www.dpi.nsw.gov.au/agriculture/livestock/sheep/health/internal/registered-drenches-sheep-worms>. Accessed 29 April 2008. Also see INFOPEST (QDPI&F). Caprimec (abamectin) was registered in Australia in 2007 for use in goats.

Appendix 5: Anthelmintics for the Control of Gastrointestinal Nematodes in Alpaca

No anthelmintics are currently registered for use in alpaca in Australia.

Appendix 6: Anthelmintics for the Control of Gastrointestinal Nematodes in Cattle

A comprehensive list of currently available cattle anthelmintics is available from the APVMA PUBCRIS data base www.apvma.gov.au. Accessed 29 April 2008. Also see INFOPEST (QDPI&F) and NSW DPI, Primefacts 419, Love and Hutchinson (2007) Cattle worm control — the basics. Available at <http://www.dpi.nsw.gov.au/agriculture/livestock/health/specific/cattle/cattle-worm-control>. Accessed 29 April 2008.

Appendix 7: Withholding Periods (WHP) and Export Slaughter Intervals (ESI) for Anthelmintics for the Control of Gastrointestinal Parasites

See APVMA website for monthly updates at www.apvma.gov.au. Accessed 29 April 2008.

Part 3 Suppliers of Commercial Reagents and Kits

SVANOVIR® O.ostertagi-Ab ELISA, Art No. 10-2940-02

Svanova Biotech AB, Uppsala Science Park, SE-751 83 Uppsala, Sweden

Phone +46 18 65 49 00

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Info@sanova.com.

www.svanova.com

Part 4 Reagents and Test Kits — Validation data

None