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Review

The detection of anthelmintic resistance in nematodes of veterinary importance

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Abstract

Before revised World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines on the detection of anthelmintic resistance can be produced, validation of modified and new methods is required in laboratories in different parts of the world. There is a great need for improved methods of detection of anthelmintic resistance particularly for the detection of macrocyclic lactone resistance and for the detection of resistant nematodes in cattle. Therefore, revised and new methods are provided here for the detection of anthelmintic resistance in nematodes of ruminants, horses and pigs as a basis for discussion and with the purpose that they are evaluated internationally to establish whether they could in the future be recommended by the WAAVP. The interpretation of the faecal egg count reduction test has been modified and suggestions given on its use with persistent anthelmintics and continuous release devices. An egg hatch test for benzimidazole (BZ) resistance is described. A microagar larval development test for the detection of benzimidazole and levamisole resistance provides third stage larvae for the identification of resistant worms. The sensitivity of these two tests can be increased by using discriminating doses rather than LD_{50} values. Details are given of a PCR based test for the analysis of benzimidazole resistance in strongyles of sheep and goats, horses and cattle. Although promising for ruminant trichostrongyles, quantitative determination of gene frequency using real time PCR requires further development before PCR tests will be used in the field. Apart from faecal egg count reduction

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tests there are currently no satisfactory tests for macrocylic lactone resistance despite the great importance of this subject. Except for treatment and slaughter trials there are no validated tests for fasciolicide resistance or for the detection of resistance in cestodes.
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Keywords: Anthelmintic resistance; Detection; *Fasciola*; Nematodes; Methods

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1. Introduction

There are only three broad-spectrum anthelmintic groups available for treatment of grazing animals for the control of nematodes. Group 1, the benzimidazoles (BZ), group 2, the imidazothiazoles (levamisole, LEV) and hydropyrimidines (pyrantel/morantel), and group 3, the macrocyclic lactones (avermectins

and milbemycins, ML), have different mechanisms of action. The salicylanilides and nitrophenols are used as narrow spectrum anthelmintics for the control of *Haemonchus contortus* in sheep and in some countries organophosphates are still marketed. No new anthelmintics with different modes of action are expected on the market in the near future. The maintenance of the efficacy of existing anthelmintics is, therefore, essential for continuing animal productivity and welfare.

In the methods for detection of anthelmintic resistance (Coles et al., 1992) the definition of resistance followed that of Prichard et al. (1980). "Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population of the same species and is heritable" (Prichard et al., 1980). This definition still adequately describes the nature of resistance.

Since the publication of the World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in 1992 (Coles et al., 1992) the importance of resistance to the three groups of broad spectrum anthelmintics has increased dramatically in nematodes of sheep and goats in many parts of the world (Wolstenholme et al., 2004). Although detailed recent surveys are frequently lacking, work in South America (Waller et al., 1996), South Africa (Van Wyk et al., 1999), Australia (Love and Coles, 2002) and the UK (Sargison et al., 2001) stress that resistance is present to all three broad-spectrum anthelmintic groups and therefore, sheep production is threatened. Even in Europe (Scotland) the first sheep farm has closed due to failure of moxidectin to adequately control *Teladorsagia circumcincta* (Sargison et al., 2005). Resistance to the MLs has become common in *Cooperia* spp. cattle in New Zealand (Familton et al., 2001), is an important problem in Brazil and Argentina (Anziani et al., 2001, 2004; Fiel et al., 2001) and is present in the UK (Stafford and Coles, 1999) and the USA (Gasbarre et al., 2004). BZ resistance has been found in *Cooperia punctata*, *O. ostertagi* and *Haemonchus placei* in cattle in Argentina (Mejia et al., 2003) and in *Cooperia oncophora* in New Zealand (Winterrowd et al., 2003). Resistance is widespread to BZs in cyathostomins of horses and there is some pyrantel resistance (Kaplan, 2002). Both BZ and LEV resistant *Oesophagostomum* sp. have recently been described in pigs but prevalence in Germany was low (2–3.5%) (Gerwert et al., 2002).

In modern pastoral farming systems the main emphasis for nematode control is to limit the number of infective larvae on pasture. This is commonly achieved by regular use of anthelmintics and other manipulations of grazing management such as treating and moving animals from contaminated sites to 'clean' pasture. In addition, more persistent anthelmintics or new delivery systems have been widely used, particularly with MLs in cattle (Rehbein et al., 2002; Geurden et al., 2004). This reduces numbers of nematodes in *refugia*, i.e. not exposed to anthelmintic (Van Wyk, 2001; Coles, 2002) and will accelerate selection for resistance. If the continued spread of resistance is to be slowed, tests are required to permit the resistance status of farms to be determined to aid in planning the optimal use of the remaining effective anthelmintics, especially for the MLs.

Until new or modified methods have been adequately evaluated in a number of laboratories and the interpretation of the tests agreed they cannot be official methods of the WAAVP. The present paper seeks to present methods that may meet those requirements after further research and to suggest where new tests are urgently required, particularly for use in cattle. Existing tests are described in sufficient detail for their international evaluation.

2. Methods

In a recent paper, Taylor et al. (2002) reviewed the available in vitro tests for the detection of anthelmintic resistance. Not all are considered to be suitable for widespread use. Two tests not in the WAAVP methods (Coles et al., 1992) are provided here, the micro-agar larval development test (MALDT) and the PCR based test for BZ resistance in ovine nematodes. The major method for the detection of resistance remains the faecal egg count reduction test (FECRT) that can be used with all anthelmintic groups. Nematode eggs are counted in faeces at the time of treatment and at defined times after treatment, the time depending on the anthelmintic group used. This test is only reliable if more than 25% of the worms are resistant (Martin

et al., 1989). A controlled efficacy test is the most reliable method of confirming anthelmintic resistance but expense usually excludes its use. Nevertheless, it is the gold standard for detecting anthelmintic resistance. Guidelines evaluating the efficacy of anthelmintics have been published elsewhere and should be adopted for investigating anthelmintic resistance (Wood et al., 1995; Duncan et al., 2002). Either naturally or experimentally infected animals can be used and the dose given should be the registered label dose rate. Where the efficacy is expected to be ≥99% anthelmintic resistance is confirmed if efficacy is <95% when calculated by comparing arithmetic means, provided that sufficient animals are in treatment groups to yield statistically significant results. However, a low prevalence of resistant nematodes may be missed as will small increases in the dose killing 95% of the worms where the registered dose rate is several fold greater than the actual dose required to remove the worms, unless some form of dose titration is incorporated into the control slaughter trial.

In vitro tests are available for the detection of resistance to group 1 and group 2 anthelmintics. Recent ring testing of the egg hatch test (EHT) in European laboratories has shown that all laboratories do not obtain the same answer with the same population of *H. contortus*. This may be due to different water samples used (distilled, deionised or tap water), the degree of cleanliness of the eggs (i.e. the amount of debris present) and the method of dissolving the sample (hydrochloric acid/water or DMSO and water or just DMSO for dilutions) and is under investigation. By using a discriminating dose, as practiced in entomology for resistance management (Roush and Miller, 1986), rather than calculating the LD_{50}, the sensitivity of the test can be enhanced substantially and quantitative estimates made of the percentage of resistant eggs in faecal samples. The discriminating dose is one that kills 99% of susceptible eggs so that essentially eggs that hatch at this concentration will be resistant. The MALDT will detect both BZ and LEV resistance (pyrantel resistance in horses) and provides third stage larvae for speciation.

A discriminating dose also enhances the sensitivity and simplicity of the test. However, this test cannot reliably be used to detect ML resistance in ovine and bovine nematodes and resistance ratios are small (Gopal et al., 1999). The test cannot be used with MLs and cyathostomins as the developing stages are insensitive. A PCR based test can be used to detect BZ-resistant nematodes of sheep, but if mutations other than those at position 200 in the β-tubulin are present, current tests will not detect the resistance. Further research is required to make these tests suitable for routine use with field samples.

3. Faecal egg count reduction test (in vivo test)

3.1. Sheep and goats

The FECRT is described for sheep and goats in Box 1. An untreated group should be used to allow for natural changes in egg counts during the test, but in many farm situations the running of controls may not be practical. Ideally 5 g of faeces should be collected from the rectum of each animal although this may not be possible with young lambs. Samples

Box 1. Faecal egg count reduction test for sheep and goats

1. Randomly distribute or distribute based on egg counts.
2. Choose animals 3–6 months of age or if older with eggs counts >150 epg.
3. Use 10 animals per group if possible.
4. Rectal sample putting 3–5 g into individual pots.
5. Count using the McMaster technique as soon as possible after collection.
6. Only store at 4 °C for 24 h if using samples for culturing.
7. Individually weigh animals and give manufacturers recommend dose orally, from a syringe.
8. Take second rectal sample at the following time periods after treatment:
   Levamisole 3–7 days.
   Benzimidazole 8–10 days.
   Macrocyclic lactones 14–17 days.
9. If testing all groups in same flock, collect at 14 days (see comments).
10. For interpretation of results, see comments.

should be put into individual labelled containers and sent promptly for counting. If nematode larvae are to be cultured for identification, samples should not be stored at 4 °C for more than 24 h as this may affect the hatching of eggs of *H. contortus* and *Cooperia* (McKenna, 1998).

Anthelmintics are best given from a syringe, if possible with a syringe extender to ensure the dose goes into the rumen, but a reliable drenching gun that has been recently calibrated can be used. Due to the temporary sterilising effect of BZs and MLs faecal samples should be collected 8–10 days after using a benzimidazole and 14–17 days after a ML. Where more than one anthelmintic type is being evaluated the longer period of 14 days should be used with the proviso that LEV may not kill all immature worms.

3.2. Egg counts

It would be useful to have an agreed standard method for counting nematode eggs in faeces as there are several methods and several variations on the original McMaster technique. The details of one modified McMaster method accurate to 50 epg was provided by Coles et al. (1992). Some laboratories use pooled faecal samples and others undertake individual counts and use these to obtain a mean value. Both approaches have advantages and disadvantages. A commercial kit (FECPAK) (www.fecpak.com), which is a McMaster technique that avoids using a centrifuge, is being increasingly used on sheep farms, but it is essential on-farm operators are trained to identify eggs. An outline of the method is given by Coles (2003) for use with faecal samples from sheep and cattle. For horse faecal samples the test is simpler to use than the McMaster technique, being more sensitive and reliable, particularly at lower egg counts (Presland et al., 2005). It is recommended that larval cultures of pre- and post-treatment samples be conducted with the FECRT (see later).

3.3. Use of half-dose ivermectin in the diagnosis of anthelmintic resistance in some ovine nematodes

Dose rates at which anthelmintics are used are set to kill the dose limiting species and this may be considerably higher than the values to remove, for example, 95% of *T. circumcincta* and *Teladorsagia colubriformis* (Shoop et al., 1993). By using a half dose ML with a FECRT earlier detection of resistance can be made. Although a full dose may work, failure can be expected to occur in the near future with continued use (Palmer et al., 2001).

3.4. Interpretation of data

For an anthelmintic to be fully effective no (resistant) worms should survive treatment following the time taken to empty the intestines (usually by 48 h). However, allowance has to be made for temporary suppression of egg production (3 days LEV, 8 days BZs, 14–17 days MLs) so no eggs should be found in the faeces after these time periods. These timings are based on best guesses. Caution has to be exercised after ML treatment where animals are maintained on heavily contaminated pasture due to the possibility of re-infection and with *C. oncophora* in cattle 17 days may be too long. Any viable eggs indicate that some resistant worms may have been present in the animals at the time of treatment. Evidence of a small percentage of survivors may indicate a resistance problem that could develop with further rounds of treatment and should be monitored. However, a greater than 95% reduction in faecal egg counts indicates that anthelmintic use should still be beneficial when used in a control program to maintain productivity. Different percentage reductions in egg counts were found, depending on the method of calculation (Mejia et al., 2003), indicating the need for standardisation of methods of calculations.

3.5. Interpretation with boluses and persistent action anthelmintics

Although some boluses have been withdrawn and use of others may be declining, the use of anthelmintic boluses necessitates some explanation of the action of the bolus and how data should be interpreted. Currently available long acting boluses for sheep are designed to act therapeutically and kill the existing burden and then act prophylactically to block the establishment of new patent infections in the host by continuing to release about 10% of a standard single dose of active ingredient per day for about 100 days. The presence of viable eggs in

faeces after administration of anthelmintic (respecting a similar time to empty the intestines as described above) is indicative of anthelmintic resistance either through survival of existing nematodes or establishment of a new infection. In itself failure of a capsule in a single animal may be due to regurgitation of the capsule, but failure in several animals constitutes anthelmintic resistance. To confirm this requires a thorough investigation involving worm count analysis of treated versus untreated animals. Alternatively, it may be preferable to recommend that a separate group of animals be treated with an oral formulation containing the same active ingredient and assessed by means of a FECRT.

Closantel has residual activity of 35 days in sheep against *H. contortus*. Both moxidectin and abamectin claim a protective period in sheep, with protection against infection with *T. circumcincta* and *H. contortus* of 35 days and *Oesophagostomum columbianum* of 28 days for moxidectin. Most MLs claim protection against re-infection in cattle. The results can be somewhat variable and are summarised in detail by Vercruysse and Rew (2002). For example the period of protection against *C. oncophora* at day 21 is 99% for doramectin given orally, 60% for ivermectin pour-on to 1% for moxidection pour-on. If eggs reappear within 50% of the claimed period of protection plus the pre-patent period of the worms, resistance must be suspected. Where the original product was a pour-on, confirmation of resistance should be undertaken using treatment with an injectable product or oral treatment, not a pour-on to eliminate possible problems with malabsorption (Sallovitz et al., 2002) unless, of course only a pour-on is available (e.g. eprinomectin).

3.6. Diagnosis of genus and species present (cattle and sheep)

The genus, and where possible the species, of the nematodes that are resistant to the anthelmintic should be determined. Third-stage larvae are therefore, cultured from the eggs in the faeces of the control and treated groups separately. The method is described in Box 2.

The egg counts can then be allocated to genera based on the results from 100 larvae and individual efficacies for each genus determined. To reduce problems resulting from possible differences in development of the species present, culture conditions should be the same for pre- and post-dosing cultures. For practical purposes a figure of 50 epg is required for the significance of a particular genus to be determined (McKenna, 1995).

Box 2. Culturing larvae for species identification

1. Collect about 50 g of faeces by combining similar sized samples from each animal in one treatment group.
2. Break up the faeces finely using a spatula. They should be moist and crumbly but not really wet. With wet faeces add vermiculite or sterilised peat moss.
3. Fill glass culture dishes (e.g. crystallising dish) with the mixture, covering but not sealing them and culture for 7 days at 22–27 °C at 80% humidity. It may be necessary to add water to maintain moisture level during incubation.
4. Either collect the larvae in a Baermann apparatus, or by suspending the mixture in water in muslin, or from standing the mixture in a petri dish containing water (see Ministry of Agriculture, Fisheries and Food (MAFF) (1986) for details).
5. Treat the larvae with Lugol's iodine and identify 100. Identification guides are given, for example, in MAFF (1986).

3.7. Cattle

The ideal is to use animals with a minimum individual count of 100 epg. This is lower than recommended for sheep as eggs counts in cattle are usually lower than those in sheep. If possible groups of 15 cattle should be used and those with no egg counts removed. Ideally only oral formulations should be used in a FECRT. Timing between treatment and the second egg counts is the same as for sheep. For ML products with persistent activity a second sample at 28 days post-treatment is recommended but this must be combined with larval cultures and interpretations can only be made for species for which persistence is claimed. If initial egg counts are below 150 epg, egg counting may

require the use of a method more sensitive than the modified McMaster technique used for sheep, e.g. FECPAK, Stoll or modified Wisconsin methods.

3.8. Horses

The ideal is to use animals with a minimum individual count of 150 epg. Group sizes will be small and control groups may not be practical but a group size of six should be used where possible. With horses being brought onto a yard it may be necessary to use single animals. Resistance is currently confined to benzimidazoles (common) and pyrantel (common in the USA; possibly due to pyrantel being used as a low daily dose, but may not be common in other countries where daily doses have not been used). Resistance has not yet been confirmed to the MLs. Bauer et al. (1986) used a reduction in egg count of less than 90% as diagnostic of resistance. However, Dargatz et al. (2000) have suggested that different cut off levels are required for different anthelmintics. Further research is clearly required in this area. Recently a significant correlation ($p = 0.025$) was found between the FECRT and EHT using a 'yes–no' answer (Wirtherle et al., 2004). Earlier investigations also found a correlation between the results of these tests (Craven et al., 1999; Várady et al., 2000) but these correlations are not very exact and further research is required including the comparison of discriminating doses in in vitro tests with FECRTs. Timing between treatment and second egg counts is the same as for sheep. The FECPAK test is probably currently the best test for use with horse samples (Presland et al., 2005). The method is summarised in Box 3.

Box 3. The FECPAK method for egg counts in horses

1. Weigh out 15–20 faeces into a grip seal plastic bag.
2. Add four volumes of water, seal the bag and thoroughly mix.
3. Pour 45 ml sample into a suitable container and add 185 ml saturated salt solution.
4. Mix contents and pour through 1 mm sieve.
5. With careful mixing fill two sides of the FECPAK slide.
6. Count eggs under both grids. One egg = 25 epg.

3.9. Pigs

It has been suggested that animals with a minimum egg count of 50 for *Oesophagostomum* spp. and a group size of 10 animals should be used but with so few cases having been investigated this is open to discussion. Resistance appears to be confined to benzimidazoles and levamisole/pyrantel in *Oesophagostomum* sp. in Germany (Gerwert et al., 2002). Sows carry the heaviest infection and should be used for anthelmintic resistance tests with this genus of nematodes. A reduction in egg count of less than 90% is indicative of anthelmintic resistance, but further research is required to see if this value should be raised to 95%. Validated in vitro tests are also required.

4. The egg hatch test

This test is for the detection of BZ resistance. The original test was described by Le Jambre (1976) and has been used with minor modifications by a number of workers (Taylor et al., 2002). Thiabendazole is used due its relatively high solubility in water. The pure chemical can be purchased from the Sigma Chemical Company (T8904). The long term stability of thiabendazole in solutions of DMSO is not known but reduction in anticipated concentrations may occur when stock solutions are diluted in water.

4.1. Collection of samples

Sensitivity to thiabendazole decreases with the age of the eggs, therefore, eggs should be used within 3 h of collection or they can be stored anaerobically (Hunt and Taylor, 1989). Anaerobic storage and isolation of eggs are given in Box 4. The method has not been checked with samples from pigs and cattle. The method for the egg hatch test is given in Box 5.

4.2. Discriminating doses

The discriminating dose is a dose that prevents the hatching of 99% of susceptible eggs. By definition,

Box 4. Anaerobic storage of eggs for Egg hatch test and isolation of eggs

1. Add faeces to a 100 ml screw-top plastic bottle containing about ten 8 mm glass beads. Fill the bottle almost full with water and shake vigorously. The contents will rapidly become anaerobic.
2. Store the bottle at room temperature. Do not refrigerate. Eggs for tests can be used up to 7 days after collection.
   Horse samples can be treated similarly but addition of 1 g glucose may increase the onset of anaerobic conditions.
3. Homogenise faecal samples until all pellets are broken.
4. Pour through a 150 μm 20 cm diameter sieve and pour the filtrate into centrifuge tubes.
5. Centrifuge for 2 min at about 300 × *g* and gently pour or suck of the supernatant.
6. Agitate the tubes to loosen the sediment, then add saturated sodium chloride solution until a meniscus forms above the tube, add a cover slip and re-centrifuge for 2 min at about 130 × *g*.
7. Pluck off the cover slip and wash the eggs into a conical centrifuge tube. With centrifuges where use of a cover slip is difficult the top layer of solution containing egg may be sucked off after centrifugation with a Pasteur pipette. Fill the tube with water and centrifuge for 2 min at about 300 × *g*.
8. Remove the water, re-suspend the eggs in water, estimate the numbers of eggs per millilitre and dilute to 100–150/100 μl.

Box 5. The Egg hatch test

1. Add 1.89 ml water to each well in a 24 well plate. This should be deionised water with a neutral pH. Then add 10 μl of thiabendazole (Sigma-Aldrich T8904) solution dissolved and diluted in DMSO to the water. Add DMSO to the control wells. Do not dilute DMSO solutions of thiabendazole in water.
2. To determine the degree of resistance use 0.05, 0.1, 0.2, 0.3 and 0.5 μg/ml thiabendazole. A single concentration of thiabendazole can be used, the discriminating dose (see text).
3. Place 100 μl of fresh eggs (less than 3 h old or anaerobically stored) in each well. Since thiabendazole may bind to debris the eggs should be as clean as possible. Incubate at 25 °C for 48 h.
4. Add two drops of Lugol's iodine to each well. Count at least 100 of the remaining eggs and hatched larvae. Either count directly using an inverted microscope or carefully wash eggs and larvae out of the well onto a microscope slide or petri dish marked with a grid and count on a compound/binocular microscope.

eggs hatching are then resistant. Discriminating doses have been established using susceptible isolates of *H. contortus*, *T. circumcincta* and *Trichostrongylus colubriformis*. Present data suggests a dose of 0.1 μg/ml thiabendazole will prevent the hatching of 99% of these species. Field tests have also demonstrated that other susceptible species (*Cooperia* and *Oesophagostomum*) do not hatch at this concentration of thiabendazole. Further evaluation to confirm these values are required so that the percentage of hatched eggs that are diagnostic of low levels of resistance can be agreed. Until laboratories are familiar and confident with this technique dose response curves should be run. Mixing of known numbers of susceptible and BZ-resistant *H. contortus* eggs has shown that 2–3% of resistant eggs can be detected using a discriminating dose (C. Yue and G.C. Coles, unpublished). The percentage of eggs hatching in the discriminating dose indicates the percentage of benzimidazole resistant eggs in the sample. It will probably not indicate eggs that are heterozygous for resistance unless the genes for resistance are dominant, but further investigation is required.

4.3. Cattle

Discriminating doses have not been established for eggs from nematodes of cattle.

4.4. Horses

Using nematodes eggs collected from semi-feral ponies that are never treated with anthelmintic and contain mixtures of small and large strongyle eggs, a

discriminating dose was established at 0.185 μg/ml. Since the frequency of genes for benzimidazole resistance in this unselected population is not known, this value could include some naturally occurring resistant worms and may be too high (G.C. Coles, unpublished).

4.5. Pigs

Discriminating doses have not been established for eggs from nematodes of pigs.

5. The microagar laval development test

Two versions of the larval development test have been used. The first to be described in adequate detail was a liquid based test (Hubert and Kerbouf, 1992). An alternative, a microagar larval development test (MALDT) is described here and is probably similar to the commercial Drenchrite® test as used, for example by Gill et al. (1995). Both tests rely on the development of eggs to L_3 larvae. Comparison of the two tests for ease of use and reliability needs to be undertaken (Box 6).

Unlike the EHT the age of eggs used in the MALDT is unimportant. The L_3 larvae can be speciated at the end of the test to indicate the species present (control wells) and those surviving anthelmintic. The test currently is only reliable for BZs and LEV. It should be noted that different concentrations of yeast extract and fungicide (amphoteracin B) are required for ovine and equine nematodes (G.C. Coles, unpublished information). The test has not been adequately evaluated for use with bovine or porcine nematodes. A commercial kit (Drenchrite®) is available and has been used, for example, for survey of anthelmintic resistance in horse populations (Young et al., 1999). Pure samples of thiabendazole, levamisole (L9756) and soluble amphoteracin B (A9528) can be purchased from the Sigma-Aldrich Company.

The discriminating doses for use in the test have been determined to be: ovine nematodes, 0.02 μg/ml thiabendazole, 0.5 μg/ml LEV and for equine nematodes (large plus small strongyles) thiabendazole 0.12 μg/ml and LEV 0.4 μg/ml. Further independent research to confirm these values is required.

Box 6. The MicroAgar larval development test

1. Extract nematode eggs from faeces as described for the egg hatch test. As long as the eggs have not started to hatch their age is not important.
2. Fill the outer rows of wells of a flat-bottomed 96 well plate with distilled/deionised water. This is to stop drying out of the test wells.
3. Put 10 μl water (control wells) or 10 μl of anthelmintic solution in the bottom of the well using 6 wells per anthelmintic concentration.
4. Add 150 μl of 2% Bacto Agar at 45 °C into each well. Allow to cool to room temperature. A repeater pipette can be used to speed the process.
5. Add 10 μl of eggs, from a stirred suspension that has been diluted with a solution of amphoteracin B. It is important that the variation in the number of eggs per well is very small. Do not use a repeater pipette as eggs precipitate very quickly. The eggs are diluted 1:1 with amphoteracin B to give a final count of 50–80 eggs per well. For ovine nematodes dilute the suspension of eggs with amphoteracin B at 0.3 mg/ml and for horses at 0.5 mg/ml.
6. Add 10 μl of yeast extract prepared as described by Hubert and Kerbouf (1984) (1 g yeast extract plus 90 ml 0.85% NaCl. Autoclave for 20 min. Add 3 ml of 10× concentrated Earles's solution per 27 ml of yeast extract). For ovine nematodes, use as described. For equine nematodes, dilute 2.5× before use. Seal the plates with tape or place in a small enclosed container with open water source for stable humidity and incubate at 25 °C for 7 days.
7. Count the numbers of live L_3 in each well. For ovine nematodes, remove larvae and speciate. With equine samples, check for the presence of large strongyles (*Strongylus* sp.).

Alternatively the more traditional approach of determining a LD_{50} or LD_{95} can be undertaken. In this case a range of drug concentrations is required.

As with the EHT discriminating doses can reduce the numbers of drug concentrations required and increase the sensitivity of the test.

6. Molecular based tests

Details of tests are only given for benzimidazole resistance as the molecular mechanisms for levamisole/pyrantel and ML resistance are currently insufficiently understood. Before the tests described are likely to be used routinely in the field they will have to be developed as real-time PCR or pyrosequencing assays. The key issue is that only when a diagnosis based on using pooled larval DNA samples can be obtained will it be possible to bring molecular resistant testing to routine use. Testing of representative numbers of single stages is prohibitively expensive. Also the available molecular tests mainly address resistance in species where the problem is widespread and in some cases may be too common to justify testing.

The most common molecular mechanism that confers BZ resistance in trichostrongyles in small ruminants involves a phenylalanine to tyrosine mutation at residue 200 of the isotype 1 β-tubulin gene (Kwa et al., 1994, 1995; Elard et al., 1996, 1999). In small strongyles in horses, and in *C. oncophora* in cattle the same polymorphism was described (Pape et al., 1999, 2003; Samson-Himmelstjerna et al., 2001, 2002b; Njue and Prichard, 2003; Winterrowd et al., 2003). However, in addition a similar mutation at codon 167 may be involved in BZ resistance in nematodes (Prichard, 2001; Pape et al., 2003) and there could be other specific mechanisms. Although poorly efficient, non-specific mechanisms such as drug transport may also confer resistance (Xu et al., 1998; Kerboeuf et al., 1999) and could even confer advantage to a worm when a novel drug is introduced.

6.1. Benzimidazole resistance diagnosis for trichostrongylid species

6.1.1. Collection of larvae, DNA extraction

DNA is prepared as a crude larval lysate from a suspension of exsheathed larvae. The concentration must be less than 1000 larvae/ml. Presence of a single larva in each microtube must be checked visually and carefully as the presence of more than one larva can lead to an incorrect genotype. Proteinase K must be inactivated before the PCR is run to allow for further amplification by *Taq* polymerase. Experimental conditions are summarised in Box 7.

Box 7. Collection of larvae and DNA extraction

1. Exsheath larvae by incubation for 5 min in a Petri dish containing 4 ml larvae suspension (less than 1000 larvae/ml) and 180 μl sodium hypochlorite (aqueous solution, about 3.5% active Cl, Rectapur®, Prolabo).
2. Individual larva in 2 μl water is pipetted under microscopical surveillance into a microtube.
3. Add 5 μl of extraction buffer (1 mM Tris–HCl, 0.1 mM EDTA, 5 mg/ml proteinase K).
4. Place microtubes at 41 °C overnight.
5. Place microtubes at 95 °C, for 20 min.
6. Store at −20 °C until molecular analysis.

6.1.2. Test procedure: species identification/ benzimidazole resistance status

Species identification relies on the polymorphism of *Rsa*I restriction enzyme sites in isotype 1 β-tubulin gene. First, two consecutive PCRs (nested-PCR) are performed on isotype 1 β-tubulin gene to amplify sufficient DNA. Then, restriction fragment length polymorphism (RFLP) of the resulting fragment is analysed with *Rsa*I to distinguish *T. circumcincta*, *H. contortus* and *T. colubriformis*. Experimental conditions are given in Box 8. This method overcomes the limitations of morphological identification of larval stages of trichostrongylid nematode species.

The principle of the BZ resistance diagnosis relies on a multiple allele-specific PCR. A set of four primers is used: two allele non-specific primers and two allele-specific primers. For *T. circumcincta*, the four primers multiplex PCR detects both alleles in the same reaction. Expected results are three fragments for heterozygotes and two fragments for homozygotes. Fragment size allows the distinction between susceptible and resistant homozygotes (see Fig. 1). Experimental conditions are summaraised in Box 9. For *T. colubriformis* and *H. contortus*, two multiplex PCR are performed for each individual: the three primers multiplex PCR detects only one allele in the reaction. Expected results are two fragments when the expected allele is present and one fragment when the expected allele is absent.

Silvestre and Humbert (2000) showed that for *T. circumcincta*, the four-primer BZ resistance diagnosis gave reliable results, although the three-primer genotyping strategy was more reliable for

Box 8. Species identification

1. Place 7 μl of digested larva in 5.25 μl of reaction mixture containing: 1.25 μl 10× buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 6.5 pmol of each primer Pn1 and Pn2, 0.5 U *Taq* polymerase (Promega, France) (see Table 1 for primer sequences).
2. Program amplification is: 94 °C for 2 min, then 20 cycles of 94 °C for 55 s, annealing temperature of 57 °C for 55 s, 72 °C for 55 s, then a final step at 72 °C for 10 min.
3. Place an aliquot of 1 μl of the [Pn1–Pn2] PCR product in 24 μl reaction mixture containing 2.5 μl 10× buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 12.5 pmol of each primer Pn3 and Pn4, 1 U *Taq* polymerase (Promega, France) (see Table 1 for primer sequences).
4. Program amplification is: 94 °C for 2 min, then 33 cycles of 94 °C for 55 s, annealing temperature of 55 °C for 55 s, 72 °C for 55 s, then a final step at 72 °C for 10 min.
5. Digest 10 μl of [Pn3–Pn4] PCR product with *Rsa*I (40 U *Rsa*I, 2 μl *Rsa*I buffer) for 1.5 h at 37 °C.
6. After gel electrophoresis in 2.5% agarose gel in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8), specific profiles are observed (see Fig. 1 for each species main fragments size).

T. colubriformis and *H. contortus* species. In all cases the multiplex PCR encompasses a non-allele specific fragment as internal standard. Several practical recommendations may be useful: "fresh" larvae are necessary to obtain reliable results. Although *T. colubriformis* larvae stored for 1 month at 4 °C were correctly typed (Silvestre, unpublished data), *T. circumcincta* larvae kept in liquid nitrogen were less efficiently amplified. Specificity of multiplex PCR may be altered by primer concentration and must be checked very precisely in order to ensure an efficient competition between primers. Although in theory, the specificity of allele amplification relies on the nucleotide in position 3′, false positives were obtained when primer concentrations were unbalanced (Silvestre, unpublished data). Known susceptible and resistant individuals used as standards should be tested to validate genotyping of unknown populations.

The present test was developed for *T. circumcincta*, *T. colubriformis* and *H. contortus* and a similar test has been applied to *C. oncophora* from cattle (Njue and Prichard, 2003). β-tubulin gene sequences do not appear to be available for other trichostrongylid nematodes. The limit of detection of BZ resistance by molecular diagnosis was estimated by Elard et al. (1999): the number of worms of one particular species to be processed in order to find at least one resistant individual ($p = 0.002$) is 100, 50, 35 and 20 for, respectively, 4, 8, 10 and 12% resistant worms in the population. In a mixed population of nematodes the allele frequency will depend on the proportion of species in the community. The presence of homozygous resistant genotypes in any species would indicate the presence of resistance.

6.2. Benzimidazole resistance diagnosis in small strongyles species

In contrast to the assays described above for the analysis of BZ resistance in trichostrongyles, the respective currently described protocols for cyathostomins do not include an internal non-allele specific PCR control. Therefore, it is important to perform separate positive PCR controls. The integrity of the DNA may be tested by the amplification of other gene sequences, e.g. ribosomal DNA.

6.2.1. Conventional allele-specific PCR

Small strongyles larval (*Cylicocyclus nassatus*, *Cylicocyclus insigne*, *Cylicocyclus elongatus*, *Cylicocyclus radiatus*, *Cyathostomum pateratum*, *Cyathostomum catinatum* and *Cyathostomum coronatum*) material is first exsheathed (Samson-Himmelstjerna et al., 1998) by incubation in 5 ml 0.1625% NaOCl in a 50-ml screw-capped (NUNC) tube at 40 °C for 5–10 min, followed by three washing steps with water. DNA is isolated with the Nucleospin® tissue kit (Macherey-Nagel, Dueren, Germany) according to the standard protocol with 1/10 standard protocol buffer volumes and DNA is eluted with 40–50 μl double distilled water. For small strongyles, the principle of the diagnosis relies on two sets of two primers. Each set consists of the same reverse primer (CN30R, non allele-specific) and one of the two allele-specific forward primers (Cn24FS or Cn25FR). Primer sequences (CN30R, Cn24FS and Cn25FR) are

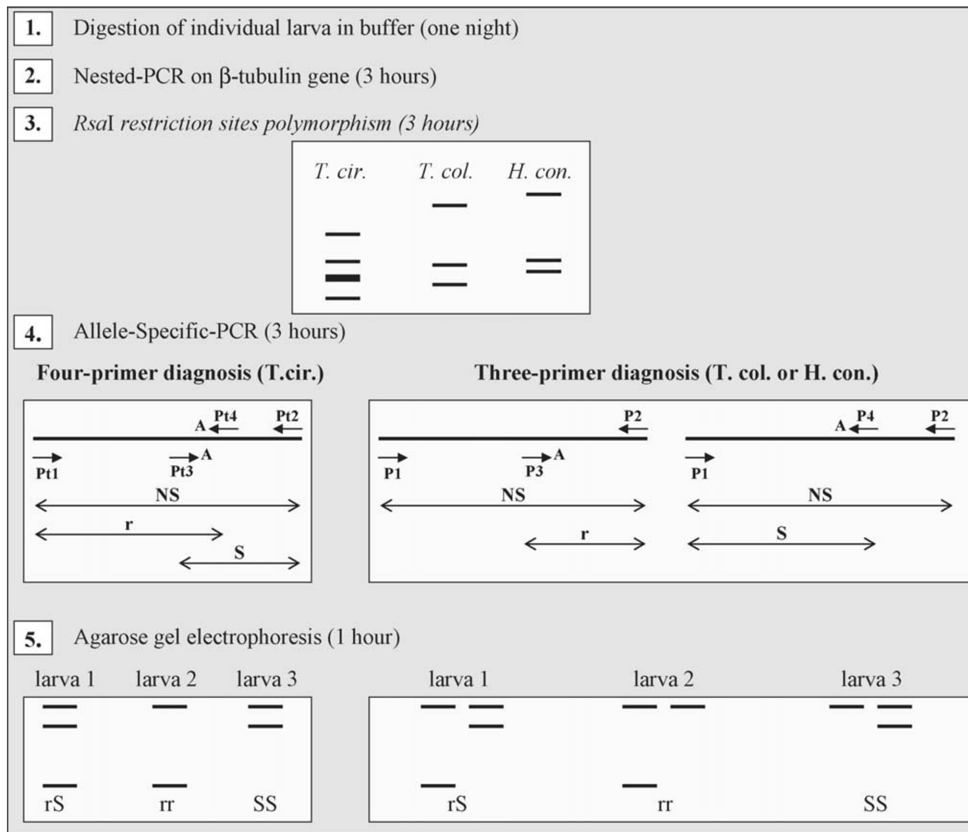

Fig. 1. Principle of the technique used for species identification and BZ resistance diagnosis in the third larval stage of the three principal trichostrongylid species of sheep: *Teladorsagia circumcincta*; *Trichostrongylus colubriformis*; and *Haemonchus contortus*. NS: non allele specific fragment; r: BZ resistance specific fragment; S: BZ susceptibility specific fragment.

presented in Table 1. The conditions for the test are given in Box 9.

Seven small strongyles species were tested with the allele-specific PCR. All resulted in the amplification of specific products (Samson-Himmelstjerna et al., 2002a). In some reactions, an additional second product of 251 bp was generated. This is presumably caused by the presence of a facultative copy of the gene, missing the 57 bp intron within the fourth exon of the cyathostomin β-tubulin isotype 1: (Samson-Himmelstjerna et al., 2002a). Even if this test is not operational for the 50 small strongyle species in horses, it is reliable for the seven most prevalent small strongyles species (Samson-Himmelstjerna et al., 2002a).

However, a survey on BZ resistance (FECRT and genotyping) in small strongyles was performed in Chile (Samson-Himmelstjerna et al., 2002b), and even if the percentage of resistant homozygous individuals was higher in treated populations, no statistically significant difference was found compared with the percentage of susceptible homozygotes (Samson-Himmelstjerna et al., 2002b). The genotyping of one BZ-susceptible and one resistant small strongyle population revealed that a decrease of the homozygous TTC/TTC genotype at codon 200 was associated with BZ resistance. However, in contrast to the trichostrongyles this genotype was still present in a major proportion of the BZ-resistant population and the TAC/TAC genotype did not increase significantly (Pape et al., 2003). The role played by mutation in codon 167 of isotype 1 β-tubulin gene in conferring BZ resistance to small strongyles is under investigation. The complete β-tubulin isotype 1 coding

Box 9. Test procedure: BZ resistance status

For *Teladorsagia circumcincta*

1. Place an aliquot of 1.5 μl of the [Pn3–Pn4] PCR product in 23.5 μl reaction mixture containing 2.5 μl 10× buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 12.5 pmol of each primer Pt1 and Pt2, 37.5 pmol of each primer Pt3 and Pt4, 1 U *Taq* polymerase (Promega, France) (see Table 1 for primer sequences).
2. Program amplification is: 94 °C for 2 min, then 33 cycles of 94 °C for 55 s, annealing temperature of 57 °C for 55 s, 72 °C for 55 s, then a final step at 72 °C for 10 min.
3. After electrophoresis in 2.5% agarose gel in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8), specific profiles are observed (see Fig. 1 for fragments size).

For *Trichostrongylus colubriformis*

1. Place an aliquot of 1.5 μl of the [Pn3–Pn4] PCR product in 23.5 μl reaction mixture containing 2.5 μl 10× buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 8.5 pmol of each primer Pc1 and Pc2, 25 pmol of each primer Pc3, 1 U *Taq* polymerase (Promega, France) (see Table 1 for primer sequences).
2. Do the same with Pc4 primer.
3. Program amplification is: 94 °C for 2 min, then 33 cycles of 94 °C for 55 s, annealing temperature of 55 °C for 55 s, 72 °C for 55 s, then a final step at 72 °C for 10 min.
4. After electrophoresis in 2.5% agarose gel in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8), specific profiles are observed (see Fig. 1 for fragments size).

For *Haemonchus contortus*

1. Place an aliquot of 1.5 μl of the [Pn3–Pn4] PCR product in 23.5 μl reaction mixture containing 2.5 μl 10× buffer, 1.5 mM $MgCl_2$, 80 μM of each dNTP, 8.5 pmol of each primer Ph1 and Ph2, 25 pmol of each primer Ph3, 1 U *Taq* polymerase (Promega, France) (see Table 1 for primer sequences).
2. Do the same with Ph4 primer.
3. Program amplification is: 94 °C for 2 min, then 33 cycles of 94 °C for 55 s, annealing temperature of 55 °C for 55 s, 72 °C for 55 s, then a final step at 72 °C for 10 min.
4. After electrophoresis in 2.5% agarose gel in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8), specific profiles are observed (see Fig. 1 for fragments size).

For small strongyles

A. Conventional PCR using single larvae
   1. Place 12 μl of genomic DNA in 50 μl reaction mixture containing: 5 μl 10× buffer, 1.5 mM $MgCl_2$, 40 μM of each dNTP, 50 pmol each of CN24FS and CN30R primer, 1.25 U *Taq* polymerase (AmpliTaq™ Gold, PE Biosystems, Weiterstadt, Germany).
   2. Do the same with CN25FR and CN30R primer combination.
   3. Program amplification is: 95 °C for 10 min, 40 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min.
   4. Presence of allele specific fragments analysed on separate lanes in 2% agarose gel stained with Gelstar® (Biozym, Hessisch Oldendorf, Germany).

B. Real time PCR using single larva
   1. Place 17 μl of genomic DNA in 25 μl reaction mixture (Brilliant core buffer® reagents, Stratagene) containing 10 and 300 nM btub mgb1 forward and reverse primer, respectively, 200 nM btub mgb T probe, 10 nM ROX.
   2. Do the same with CN25FR and CN30R primer combination.
   3. Program amplification is: 95 °C for 10 min, 40 cycles 95 °C for 15 s, 64 °C for 60 s.
   4. The cycle threshold (ct)-values are calculated based on the normalized baseline corrected fluorescence (dRn) (Mx4000 Multiplex Quantitative PCR System® Stratagene).

sequences were described by using single worm cDNA of six cyathostomin species from a BZ-resistant small strongyle population. Surprisingly, all sequences showed TTC at codon 200, whereas for each species TAC was found at position 167 (Drogemuller et al., 2004). The allele frequencies in the latter polymorphism still need to be further investigated in BZ-susceptible and resistant cyathos-

Table 1
Primers sequence for each polymerase chain reaction of the molecular diagnosis of benzimidazole resistance for trichostrongylid nematodes

Species	Primer name	Primer sequence
ND[a]	Pn1	5′ ggC AAA TAT gTC CCA CgT gC 3′
	Pn2	5′ gAA gCg CgA TAC gCT TgA gC 3′
	Pn3	5′ gTg CTg TTC TTg TTg ATC TC 3′
	Pn4	5′ gAT CAg CAT TCA gCT gTC CA 3′
Teladorsagia circumcincta	Pt1 forward primer	5′ ggA ACA ATg gAC TCT gTT Cg 3′
	Pt2 reverse primer	5′ gAT CAg CAT TCA gCT gTC CA 3′
	Pt3 resistant allele primer	5′ TTg gTA gAA AAC ACC gAT gAA ACA TA 3′
	Pt4 susceptible allele primer	5′ gTA CAg AgC TTC ATT ATC gAT gCA gA 3′
Trichostrongylus colubriformis	Pc1 forward primer	5′ ggA ACA ATg gAT TCC gTT Cg 3′
	Pc2 reverse primer	5′ ggg AAT Cgg Agg CAA gTC gT 3′
	Pc3 resistant allele primer	5′ CTg gTA gAg AAT ACC gAT gAA ACA TA 3′
	Pc4 susceptible allele primer	5′ ATA CAg AgC TTC gTT ATC gAT gCA gA 3′
Haemonchus contortus	Ph1 forward primer	5′ ggA ACg ATg gAC TCC TTT Cg 3′
	Ph2 reverse primer	5′ ggg AAT CgA Agg CAg gTC gT 3′
	Ph3 resistant allele primer	5′ CTg gTA gAg AAC ACC gAT gAA ACA TA 3′
	Ph4 susceptible allele primer	5′ ATA CAg AgC TTC gTT gTC AAT ACA gA 3′
Small strongyles	CN30R reverse primer	5′ AgC AgA gAg ggg AgC AAA gCC Agg 3′
	Cn24FS susceptible allele primer	5′ ggT TgA AAA TAC AgA CgA gAC TTT 3′
	Cn25FR resistant allele primer	5′ ggT TgA AAA TAC AgA CgA gAC TTA 3′
Small strongyles real-time PCR	btub mgb1 forw	5′ AATgCTACCCTATCCGTTCATCA 3′
	btub mgb1 rev	5′ CAAATATCATAgAgAgCTTCATTgTCAAT 3′
	btub mgb T	5′ FAM-AATACAgACgAAACTTTCTg 3′
	btub mgb A	5′ FAM-AATACAgACgAAACTTACTg 3′

[a] Not determined.

tomin populations. Recently the β-tubulin isotype 2 sequences of the two cyathostomin species were identified and found to show TTC at both codon 167 and 200 (Clark et al., 2005). However, since these polymorphisms are believed to also be related to BZ resistance in trichostrongyles (Prichard, 2001), they will as well have to be addressed in cyathostomins.

6.2.2. Real-time allele-specific PCR

One forward and one reverse primer are used within two different allele-specific TaqMan minor-groove-binder (MGB)-probes in separate reactions. The sequence of the primer probes are given in Table 1. The probe 'btub mgb T' is specific for the BZ susceptibility related TTC-allele, while 'btub mgb A' detects the resistance related TAC-allele. For each larva two replicates are analysed. For details see Box 9.

The allele-specific real-time PCR procedure reproducibly allowed the reliable genotyping of single adult and larval DNA samples as with the conventional allele-specific PCR (Samson-Himmelstjerna et al., 2003). A BZ-resistant field population tested by this method showed similar genotype frequencies as populations that were experimentally selected for BZ resistance (Pape et al., 2003). Since no post-PCR steps are required this procedure allows a significant increase in sample throughput. However, as already discussed, in small strongyles the role of the β-tubulin codon 200 polymorphism is not linked as closely with the phenotype of BZ resistance as found in trichostrongyles. Only moderate changes in allele frequencies were observed which were not distinguishable by real time PCR. Therefore, the quantitative analysis of TTC/TAC copy numbers in samples of pooled parasites was not accurate enough to reliably characterize the respective population. The standard deviations within the standard curve samples, together with those of the sample replicates, accumulated so that the calculated copy number results showed coefficients of variance of more than 50%.

7. Liver fluke and tapeworms

The main problem at present with resistance appears to be with triclabendazole in *Fasciola hepatica*, although failures of closantel have been reported in Australia. Benzimidazole resistance in tapeworms appears to be occurring in sheep (Southworth et al., 1996) and praziquantel may be showing reduced efficacy in sheep in New Zealand. The only tests available are dose and slaughter trials, e.g. Coles and Stafford (2001). FECRTs have not been standardised for either tapeworms or flukes. This is complicated by development and egg laying by immature flukes not killed by several fasciolicides that are only effective against adult fluke and, with tapeworms, by the destrobilation rather than removal of the scolices. With tapeworms (*Anoplocephala perfoliata*) in horses, egg counts are not very sensitive measures of infection (Proudman and Edwards, 1992). There are no in vitro tests for resistance in flukes or tapeworms. The molecular basis of resistance is not known for any fasciolicides or cestodicides so no molecular based tests are available. A miracidial test has been described for praziquantel resistance in schistosomes of domesticated animals, but further research is required before it can be used in the field situation (Kenworthy et al., 2003).

8. Discussion

Given the huge impact that anthelmintic resistance can and will have on ruminant production and welfare (Coles et al., 2004) and the health of horses, the ability to reliably detect resistance and to compare data between countries and regions is vital. It is also essential to be able to give the best advice to producers on control strategies. Therefore, it is disappointing that relatively little progress has been made on new and improved tests to detect anthelmintic resistant helminths since the first WAAVP methods were published in 1992 (Coles et al., 1992). This is a direct result of the lack of interest in funding agencies in supporting this type of research. Hopefully this is being remedied and it will prove possible to produce standard operating procedures (SOP) for running and interpreting a range of tests to detect resistance. Some developments will require a much greater understanding of the molecular biology of anthelmintic resistance, others only require agreement on which are the best ways of running faecal egg counts in particular species and the statistics used to interpret the results.

8.1. Faecal egg count reduction test

It would be of value to decide which egg counting procedure is best for which animal faecal samples. For example whilst the McMaster test, which was developed for egg counts in sheep, is probably the best for single sheep samples, perhaps the FECPAK test is best for pooled samples. FECPAK certainly is better than the McMaster test for horse samples (Presland et al., 2005) and since it does not involve use of a centrifuge can be used on the farm by any operator who can reliably identify nematode eggs. More information is needed on: what are the minimum egg counts required in animals for their incorporation into trials and how do they differ between species? Do we really need a control group in sheep flocks and can composite samples be used for on farm test for apparent drench failures? It would simplify the testing and reduce costs if information on these aspects were available. An agreement on what level of egg reduction indicates resistance for particular anthelmintics and animal species is also required as is the optimal time for sampling after treatment with MLs. Agreement is also required on interpretation of data following use of boluses of persistent anthelmintics (primarily MLs). In horses a reduction in egg reappearance period is likely to be the first indication of developing resistance to the MLs and this period will probably differ between young animals and adults. Whilst in sheep, egg counts and worm numbers may correlate reasonably well, egg production in *T. circumcincta* in sheep can be density dependent and the problems with faecal egg counts and numbers of adult *Nematodirus* sp. are well known. The relationship between egg counts and worm numbers are not so clear in cattle. How will this complicate the FECRT, particularly where the results are near the cut off point? It is also important to know the species of nematode involved if eggs are passed after treatment. Usually identification of larvae can only be made to the level of genera and even then can be difficult. The problem of detection of ML resistance with faecal egg

counts in *Parascaris equorum* in horses needs to be discussed and agreed. This is a practical problem in a number of countries. How long after treatment should the second egg count be made? Validated molecular tests for identification of species would be of value. There is also debate on the statistics that should be used to analyse egg counts. Just how sensitive are FECRTs? One way of overcoming these problems is to run in vitro tests, but how do results from the FECRT relate to data obtained from in vitro tests?

8.2. Egg hatch test

The problems encountered in Europe in obtaining similar results from the EHT stress that details, such as the source of the water used, are very important and that ring testing is required before a SOP can be issued. Standardised tests are not only required for nematodes of sheep and goats, but as BZ resistance increases in cattle it would be of value here. Why are values for LD_{99} higher in horses using unselected populations of nematodes than for ovine nematodes? Therefore, how are EHTs on equine nematodes to be interpreted? Since using discriminating doses can increase sensitivity of the test, agreed values and their interpretation are required. However, it is essential that laboratories using this technique can obtain the same results with sensitive isolates. An egg hatch test cannot be used with *Nematodirus* sp. but a standardised egg embryonation test should be of value. Would it work with *Ascaris* and other nematodes that do not hatch before ingestion?

8.3. Larval development test

There are currently two larval development tests of interest, the liquid based test described by Hubert and Kerbouf (1992) and the agar based test of Gill et al. (1995). Is one easier to use than the other? Whilst they appear to work for BZs and LEV in ovine and equine nematodes they do not appear to work with MLs and have not been produced for use with bovine nematodes. As for the EHT large amounts of data needs to be collected before a SOP and its interpretations can be agreed. In particular the relationship needs to be determined between these standardised tests and the FECRT. Although the LDT will work for BZ resistance, it appears not to be as satisfactory as the EHT (G.C. Coles, unpublished). Whether the EHT and LDT will detect the presence of 'sr' genes (heterozygotes) remains to be established. Of course, these tests will not work with eggs that do not hatch so how is resistance to LEV to be detected in these species? A larval migration test has been suggested for ML resistance (Gatongi et al., 2003) but how will it work in mixed species and will it work equally well for ovine and bovine nematodes?

8.4. Molecular based tests

The problem with all molecular based tests is to be sure that the mutation associated with resistance is the only mutation permitting resistance to the drug under investigation in a particular species. This is well illustrated by there being more than one mutation for BZ resistance in certain ovine nematodes. If, as suspected, both isolates and different species of nematodes have different mechanisms for avoiding the action of MLs, the problem of developing and using probes is increased greatly. However, as there are no proven reliable in vitro tests for ML resistance, the need for molecular based tests is very great.

Currently the only available molecular tests are for BZ resistance in some ovine nematodes and possibly one bovine nematode (*C. oncophora*). As single larva or worm genotyping is very laborious and relatively expensive, before molecular tests will be of practical use in the field they must be developed for real time PCR or pyrosequencing. However, BZ resistance in most parts of the world is so common in ovine and caprine nematodes that it is doubtful whether sensitive tests for resistance are now relevant, except for research into how management strategies can slow the development of resistance. However, it could be very useful with bovine nematodes. Again, as with in vitro tests, ring testing is required.

8.5. Trematodes and cestodes

There are no validated tests, no agreement on interpretation of the FECRT, no in vitro tests and no information on the molecular basis of fasciolicide or cestodicide action or resistance. Clearly a large amount of research is required.

Development of new or modified tests will be of value both in the veterinary and medical fields.

Although resistance to anthelmintics in nematodes, trematodes and cestodes of humans is not currently a significant problem, its potential is very large and needs to be addressed (Albonico et al., 2004).

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