Veterinary Parasitology 329 (2024) 110187

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.): Third edition of the guideline for evaluating efficacy of anthelmintics in ruminants (bovine, ovine, caprine)

ARTICLE INFO

Keywords
Anthelmintic
Bovine
Caprine
Efficacy
Guideline
Helminths
Ovine
WAAVP

ABSTRACT

This guideline is aimed at those who are involved in the assessment of anthelmintic efficacy in ruminant livestock species (bovine, ovine and caprine). The intent is to provide a framework that can be adopted worldwide for the testing of anthelmintics in ruminants, such that studies carried out in different countries can be compared and thereby unnecessary duplication can be reduced. Recommendations are made for the selection, housing and feeding of study animals, the type of studies required, the method used to conduct those studies, the assessment of results and the standards for defining anthelmintic efficacy.

1. Introduction

The World Association for the Advancement of Veterinary Parasitology (WAAVP) guideline is intended for the evaluation of the efficacy of anthelmintics in farmed ruminants (bovine, ovine, caprine, cervine and other groups). By following this guideline, researchers can generate meaningful and complementary data which are scientifically sound and globally recognized. The guideline seeks to update the one published by Wood et al. (Wood et al., 1995). It should be read in conjunction with the WAAVP General Guidelines (Geurden et al., 2022), which describe the general principles of anthelmintic efficacy evaluation. Here, the authors seek to expand and clarify the recommendations that relate specifically to ruminants and include procedures that apply to:

1. Artificially infecting animals with targeted helminths (see Section 5).
2. Sourcing animals with natural infections of targeted helminths (see Section 5).
3. Characterizing helminths by stage of development, source and anthelmintic sensitivity/resistance (see Section 4).
4. Assessing both therapeutic and protective (prophylactic, preventative) efficacies of a product (see Sections 2 and 3).
5. Collection, counting and species identification of helminths in ruminant hosts; *postmortem* (worm burden), faecal egg counts (FEC) and faecal larval counts (FLC) (see Section 6). Suggestions for determining the statistical validity of study data are discussed in detail in the WAAVP General Guidelines (Geurden et al., 2022).
6. Documentation of all study activities, observations, and data sets (see Section 4).

Compliance with this guideline can help to reduce the number of studies and experimental animals required to generate the data needed to evaluate efficacy of anthelmintics.

The guideline was developed by researchers with first-hand experience of carrying out efficacy evaluation studies and the recommendations are based on their accumulated knowledge. It is, however, intended to be flexible, as the variable interaction between host, parasite, husbandry and product in different countries and regions may require some study-specific adjustments.

All studies should undergo an appropriate animal welfare evaluation-assessment and national ethical review prior to their initiation.

2. Standards for establishing anthelmintic efficacy

In order, to be considered effective in the treatment and/or prevention of helminth parasites, every new anthelmintic product should adequately reduce the targeted helminth population or fulfil a specific niche not covered by currently used products. Contrary to the previous edition of the ruminant anthelmintic efficacy guideline (Wood et al., 1995), the minimum level of efficacy acceptable for a new anthelmintic is now set at $\geq$ 90%, (unless the new anthelmintic provides a significant level of efficacy for which there is currently none). To be considered efficacious, the new anthelmintic must remove $\geq$ 90% of the targeted helminth populations in treated groups relative to the corresponding untreated control groups. Levels of efficacy greater than the minimum are obviously most desirable, as the likelihood of delaying the development of resistance (compound sustainability) is greatly increased with any elevation in initial efficacy (Leathwick and Besier, 2014).

Both initially, as well as after changes in formulation of a previously registered product, the level of efficacy must be demonstrated in dosage determination and dosage confirmation studies, by the controlled study method (see Section 3). Only the controlled study method is

https://doi.org/10.1016/j.vetpar.2024.110187
Received 3 April 2024; Received in revised form 15 April 2024; Accepted 15 April 2024
Available online 18 April 2024

recommended to demonstrate the anthelmintic efficacy in ruminants. The critical study method (collection, quantification and identification of worms expelled intact from treated and untreated hosts) is not generally considered appropriate for ruminant helminths (Geurden et al., 2022).

With the controlled study method, the efficacy of an anthelmintic is determined by comparing the parasite burdens in randomised groups of treated and untreated animals that are expected to have similar parasite numbers and species mix of helminths prior to treatment. This is the most reliable method for evaluating anthelmintic activity in ruminants and is recommended for both dosage determination and dosage confirmation studies (see Section 3). It is recommended to use a method that ensures that study staff are masked/shielded from treatment/non-treatment of individual animals. This can be done by using a coded placebo or by using only dedicated study staff to administer the treatment and who then take no further part in data collection.

The following formula expresses the percentage efficacy (%E) of a drug dosage against a given parasite population (species, genus and stage of development). The geometric mean worm burden of the treatment group (T) is compared to the geometric mean worm burden of the untreated control group C as follows:

%E = 100 x ([Mean of C – Mean of T] ÷ Mean of C)

The geometric mean of worm burdens is used to provide an appropriate indication of the percentage reduction due to treatment, as it most accurately reflects the distribution of helminth populations within groups of animals, especially when counts within a group are not normally distributed, as is generally the case with helminth worm burdens (Note, some jurisdictions require efficacy calculated using both the arithmetic and the geometric mean and the use of confidence intervals).

3. Types of studies required

Anthelmintic evaluation studies (dosage determination, dosage confirmation and clinical field studies) are conducted sequentially to determine, confirm, and field-test the spectrum of effective activity and the appropriate effective dosage of an anthelmintic for the targeted adult and larval parasites. These studies also serve to evaluate the safety of a new product under various climatic conditions, environments and animal husbandry systems. Whilst this section applies equally to all helminth species, the examples cited below are more relevant to gastrointestinal nematodes (GIN). Additional specific guidance relevant to other helminths can be found in Section 7. Structured evaluation studies are also indicated for additional documentation of anthelmintic activity such as:

1. Efficacy against parasites documented to be resistant to current anthelmintics.
2. Persistence of anthelmintic activity.
3. Additive, synergistic or antagonistic effects of anthelmintic combinations. Please refer to The WAAVP publication on combination products by Geary et al. (Geary et al., 2012) for further information.
4. Changes to formulation or directions for use.

The studies discussed in this paper are either controlled studies using animal necropsies and parasite enumerations, or field studies using FEC or FLC based on identification of eggs and/or larvae identified after coproculture or other biomarkers for worm infection. Biomarkers such as packed cell volume (or other measures of anaemia), serum enzyme levels, faecal antigen ELISA or growth rate can be used to provide further indirect/peripheral evidence of anthelmintic activity.

Parasites are identified by genus or species and, if possible, by stage of development and/or characterization of inhibited development (arrestment). A list of major helminth parasites in ruminants is presented in Table 5. Measuring the efficacy against adult parasites is relatively straightforward, as the treatment is given when the parasites are fully developed. Measuring of efficacy against larval stages is more challenging because non-arrested larval stages are generally of short duration and the efficacy levels of anthelmintics may exceed the period during which a non-arrested developmental stage is present. However, the evaluation of efficacy against arrested larval stages is much more feasible and of greater veterinary significance.

3.1. Dosage determination studies

Dosage determination studies are controlled studies conducted to determine the effective dosage of a product for each parasite species and developmental stage to be claimed for in their spectrum of product activity. These studies are also designed to establish the dose-limiting parasite species or developmental stage that requires the highest dosage rate to eliminate over 90% of helminths from treated animals. Dosage determination studies should be carried out with the final formulation and by the intended route of administration (Geurden et al., 2022). Studies with mixed (multi-species) infections are encouraged to reduce the total number of animals used.

Animals with similar physical characteristics (age, weight, etc.) are used. Artificial infections are best used for these studies and ideally, the animals will be reared free of other GIN exposure. Animal numbers are equal for each of the groups, and the control group must have a minimum of six animals adequately infected with the parasite(s) of interest at the time of necropsy (Geurden et al., 2022). The required groups are the control group (untreated), and groups treated with 0.5, 1.0, and 2.0 times the assumed effective dosage (ED) (Table 1). The animals can be infected with as many of the targeted parasite species as possible (caution – too many species inoculated at the same time can lead to large variations in the worm burden of individual species). Parasite infections in each study should be induced. However, natural infections are acceptable if no induced infection model is available (see Section 5). Parasite populations in these studies must be characterized by suspected/documented stage of development at treatment and the stage of development at necropsy (see Table 6).

Determining the efficacy of products with more than one active ingredient (combination or dual active products) can be a special case and the reader is advised to refer to the WAAVP guideline on anthelmintic combination products by Geary et al. (Geary et al., 2012).

3.2. Dosage confirmation studies

Dosage confirmation studies are controlled studies with typically two treatment groups per study (in some circumstances, there can be more than two groups, see Section 3.4): the untreated control group and the group receiving the proposed treatment regimen (Table 2) as determined by the dosage determination studies outlined above. They must be performed with the formulation, dose rate and means of delivery that is proposed for final authorisation. Dosage confirmation studies are conducted to confirm that the labelled dose for a product (as determined in the dosage determination studies) is an effective dose (ED) that

Table 1
Outline of a typical induced infection, dosage determination study for a helminth species with a relatively short pre-patent period (<24 days) and a product that has a therapeutic efficacy within 7 days.

Treatment group	Infection day*	Treatment day	Treatment dose**	Necropsy day
1 (Half dose)	0	24	0.5x	31
2 (Full dose)	0	24	1.0x	31
3 (Double dose)	0	24	2.0x	31
4 (Control)	0	none	0.0x	31

* Day of induced infection
** Presumed targeted effective dose = 1.0 x full dose

Table 2
Outline of a typical induced infection, dosage confirmation study for a helminth species with a relatively short pre-patent period (<24 days) and a product that has a therapeutic efficacy within 7 days.

Treatment group	Infection day**	Treatment day	Treatment dose	Necropsy day
1 (ED)*	0	24	1x	31
2 (Control)	0	None	Nil	31

* Effective dose (ED)
** Day of induced infection or final day of natural infection challenge

eliminates ≥90% of all indicated parasites.

Both natural infections and induced infections can be used for dosage confirmation studies, the latter especially for rare helminths or when stage-specific drug efficacy is evaluated. Targeted (indicated) helminths must be identified and quantified in sufficient numbers in at least six control animals at necropsy. This may require the inclusion of more than the six animals per group in the study (Geurden et al., 2022).

Dosage confirmation studies can be conducted with animals that have multiple/mixed infections (induced and/or natural infections). Further assurance that the study animals are infected with the parasite species and stages targeted in the study can be obtained by pre-treatment necropsy and parasite quantification of at least 2–3 representative animals (Geurden et al., 2022). This examination should include the recovery of adults and immature stages *postmortem*. Additionally, or alternatively, FEC and coprocultures, which allow identification of species/genus by morphological and/or molecular methods, can demonstrate at least some of the species present (see Section 6).

3.3. Field studies

Field studies (also known as clinical studies) are designed to evaluate the final product when used according to the manufacturers' label instructions under various contrasting field, husbandry, climatic, host and parasite populations, to document further the safety of the product, its anthelmintic efficacy, and to obtain productivity data. These studies use 'biomarkers of helminth infections' (e.g. FEC with coprocultures to produce larvae for morphological or molecular identification) rather than parasite counts to demonstrate the anthelmintic effect of a product (Geurden et al., 2022). Generally, animals are not necropsied in these field studies (except in cases of unforeseen mortality, or to document parasite loads and species composition of parasite challenge using tracer/sentinel animals), as this allows for the inclusion of a larger number of animals. Efficacy can be assessed by comparing pre-treatment with post-treatment FEC (with coproculture for larval identity), provided that the study is of relatively short duration, expected treatment persistence is similar for all target species and it is conducted at a time and place where the infection pressure is unlikely to change during the study. However, the inclusion of an untreated control group makes it possible to take unforeseen events into account when evaluating anthelmintic activity. With field studies to determine persistent efficacy, it is always advisable to use a control group that allows monitoring of the infection exposure rate during the study. The previous recommendation that at least 25% of the number of animals used in the treated group should be used in the control group no longer applies but the decision on the size of the treatment/control group should be made in consultation with a statistician. The control group should be at least as large as the treatment group to obtain meaningful data (Torgerson et al., 2005). The size of the treatment group should be at least 10 but chosen to maximize the chances of determining faecal egg count reduction (FECR) above the targeted efficacy threshold with a 95% confidence interval.

Additional items relative to the conduct of field studies include, but are not limited to:

1. The product must be evaluated in relation to instructions/ prescriptions on the anticipated label (dosage, formulation, route of administration, etc.).
2. Each study should consist of a statistically valid number of animals (Geurden et al., 2022). The study animals should be held on one agricultural facility and treatment group numbers should be the same in each replicated group, with ideally a minimum of 10 per group.
3. All animals in a study must be managed and pastured in the same way. If multiple pastures are to be used in studies that include control groups, then each homogeneous pasture must contain complete sets of replicates.
4. Certain conditions must be met prior to the initiation of the study:
   a. That the animal keeper is fully knowledgeable of, and agreeable to, all study requirements (observations, records, animal handlings, observation times, withdrawal times, visitations, etc.).
   b. That the animals are sufficiently infected with as many of the targeted parasites as possible, to provide data that can be successfully analysed statistically using optimal numbers of animals.
      5. This guideline does not recommend any particular type of faecal egg counting technique. Individuals have their own preferences and laboratories are not usually equipped for all methods available. However, we make a few general points relevant to the guideline based on the more detailed discussions on the use of FEC that can be seen in Kaplan et al. (2022) and Nielsen (Nielsen, 2021):
      a. Some faecal egg counting methods have a higher detection rate/sensitivity than others. Thus, the multiplication factor used to convert the number of eggs counted under the microscope (the raw egg count) to the actual number of eggs present in the sample (FEC) varies from one technique to another.
      b. It is essential that operators follow standard operating procedures and implement sound quality control to ensure both accuracy and precision/repeatability (Nielsen, 2021). Even in the hands of experienced, competent individuals, results can vary widely between individuals and laboratories. There is typically less variation with techniques that rely on counting eggs in fixed volume chambers (e.g., McMaster and Flotac) as there is less manual dexterity and judgement required. Therefore, methods using counting chambers are preferred for use in anthelmintic studies (Nielsen, 2021), provided that the detection sensitivity is appropriate for the level of FEC anticipated.
   c. It is accepted that there can be a wide range of FEC within a group of similar aged animals sharing a similar parasite exposure history. The larger the group size, and the higher the mean FEC, the lower the requirement for high detection rate methods.

Regardless of the method chosen, FEC should be performed 10–14 days before treatment to allow time for individual coproculture results in those cases when the worm species/genera present need to be identified by larval morphology (see Section 6).

Alternatively, ruminant nematodes can be identified using molecular methods. Thanks to advances in this field, it is now possible to identify species at any stage of development (eggs, larvae or adults). This is done after extraction of genomic DNA and PCR amplification with primers targeting the gene of choice (Gasser et al., 1993). So far, different regions in the Internal Transcribed Spacer-2 region (ITS2) of the ribosomal RNA gene array in nematodes have mostly been targeted. In general, the ITS2 provides sufficient variability for species/genus identification of strongyles in ruminants, but other genes can of course also provide additional information if required.

The identification and conformation of the GINs present is then performed either directly with specific primers covering the regions of interest or, if necessary, with universal primers followed by Sanger sequencing (e.g. (Gasser et al., 1994; Wimmer et al., 2004; Bisset et al., 2014). There are several protocols for species/genera identification in

larval cultures covering both the major GINs in small ruminants (e.g., (Roeber et al., 2017; Elmahalawy et al., 2018; Santos et al., 2020) and cattle (e.g., (Höglund et al., 2013; Roeber et al., 2017; Baltrušis et al., 2019). Different experimental setups on different technical platforms can be used, allowing either semi-quantitative (i.e. qPCR and variations thereof) or absolute quantification of nucleic acid target sequences (i.e. digital PCR).

The most recent advance in the study of nematode biodiversity is the introduction of deep amplicon sequencing of so-called nemabiomes (Avramenko et al., 2015). This is a powerful DNA barcoding method that, in contrast to the methods mentioned above, utilises the advantages of primers that cover the entire ITS2. To date, metabarcoding has been performed on a variety of nematodes of veterinary interest including both those of sheep and cattle (e.g., (Avramenko et al., 2017; Avramenko et al., 2018; De Seram et al., 2022; Halvarsson and Höglund, 2021; Queiroz et al., 2020; Francis and Slapeta, 2022). Although the relative abundance of multiple species can be detected with high precision using metabarcoding in a single reaction, a pre-PCR step is usually required, making it difficult to compare the amounts of each species in different samples (Redman et al., 2019).

As indicated above the molecular tools are not limited to larval cultures. Accordingly, protocols have been developed for direct DNA extraction of nematode eggs in faeces (e.g. (Sweeny et al., 2011; Roeber et al., 2013; Höglund et al., 2019). Some kits used for DNA extraction of parasite eggs in faeces have been evaluated by Högberg et al. (Högberg et al., 2022).

The faecal sampling is carried out on day −1 or 0 (either suffices for 'day of treatment' count). The frequency and duration of post treatment sampling are based on the claims for drug efficacy. For therapeutic evaluation, faecal samples should be collected at an appropriate time post-treatment that allows sufficient time for drug activity, and resumption of oviposition by female nematodes not removed by treatment (if applicable) but excludes the establishment of patent infections by post-treatment challenge. To substantiate a claim of persistence or prophylaxis, the above faecal examination timings should be followed, as well as further observations at appropriate intervals until the end of the claimed period of sustained reduction in FEC. Multiple observations may be required if persistence is different for different parasite species.

3.4. Studies designed to document efficacy of a compound against drug resistant populations

The efficacy of anthelmintics against resistant populations of helminths are usually demonstrated in the field by the faecal egg count reduction test (FECRT) or by *in vitro* tests (Kaplan et al., 2022). However, we include this section in the guideline as the definitive test to determine efficacy against resistant populations is always a controlled dosage confirmation study. These studies are conducted with a pool of animals infected naturally with parasites known to be resistant to an existing product or class of compound, or with isolates from the field used as the source of induced infections for resistant helminths. As a rule, a normal dosage confirmation study is conducted with a control group and a treated group. However, a third group treated with a reference product (known to have reduced efficacy against resistant populations) should be added to confirm the degree/level of resistance within the parasite population. The products tested should be of market formulation (current or intended) and administered at the correct dose level proposed for the intended veterinary product. An appropriate level of infection with the target parasites must be present in a minimum of six control group animals at necropsy.

Whether induced or natural infections are used, documentation of the anthelmintic resistance status of the worms infecting the study animals needs to be provided (see (Denwood et al., 2023).

Other characteristics that should be documented are:

1. Is the resistance against a single molecule, across a family of molecules (side resistance), and/or shared by molecules of different classes (cross resistance)?
2. Which parasite genera/species are resistant?
3. References/details of previous relevant studies such as results of FECRT, *in vitro* tests or PCR results.

3.5. Studies for documenting persistent anthelmintic activity

Some anthelmintics may have prolonged efficacy; in this case, there is a post-treatment period during which the treated animal is "protected" from a new parasite challenge (prophylactic effectiveness). This persistence/prolonged effect may be due to the residual nature of the anthelmintic, from a slow-release capsule in the rumen or from a depot either at the injection site or deposited in fatty tissue after treatment. Tables 5 and 6 illustrate how these dosage confirmation studies can be conducted using either induced or natural infections respectively. Dosage determination studies would have previously been carried out with the candidate product to establish the effective dose and pharmacokinetic (PK) and pharmacodynamic (PD) data may provide insight into how long the efficacious/persistent activity of the product is likely to last.

Helminth-free animals are recruited to the study, randomly assigned to treatment groups and housed in their groups under conditions that prevent accidental helminth infections.

For persistent activity studies using induced infections, it is best to stagger treatment days so that each study group is treated at each of the relevant intervals (e.g., every seven days) prior to infection and all animals are infected with the same batch of larvae on the same day. This reduces the potential problem that stored larvae, even when kept under ideal conditions, tend to become less infectious over time. The study schedule outlined in Table 3 is then followed with infections and necropsies carried out as described (Sections 5 and 6).

Table 4 illustrates a study protocol to determine persistent anthelmintic activity in naturally infected animals.

After treatment, the animals can be kept together on a contaminated pasture during the study if the treatment is oral or parenteral. However, if treatments can be passively transferred between animals, such as with a pour-on preparation, then the pasture must be sub-divided just before treatment and the treatment groups must graze separately. An alternative method is to separate the study groups for the usually short period during which passive transfer is possible (if known) and then graze them together.

An alternative method to staggered treatment followed by natural challenge, as outlined in Table 4, is to simulate the natural challenge with daily induced infection with a single or known mixture of helminth species. In this way, the persistence of efficacy by species can be assessed.

As with all controlled studies, the helminths claimed must be present at levels that can be quantified as sufficient at necropsy of at least 6 control animals.

Table 3
Induced infection study protocol to document anthelmintic efficacy for a product envisioned to have an effective persistence of up to 42 days after treatment.

Treatment Group	Treatment Day	Infection Day	Necropsy Day	Persistence period
1	0	42	66	42
2	14	42	66	28
3	21	42	66	21
4	28	42	66	14
5	35	42	66	7
6	42	42	66	0
7	none	42	66	nil

NB. If the persistence of efficacy is already known and the study is seeking confirmation, then many of the above groups can be removed.

Table 4
Natural infection study protocol to document anthelmintic efficacy for a product envisioned to have an effective persistence of 49, 56 or 63 days after treatment using grazing animals exposed to natural re-infections.

Treatment group	Treatment day	Challenge days[a]	Necropsy day*	Persistence period tested (days)
1	None	–3–112	140	nil
2	49	–3–112	140	63
3	56	–3–112	140	56
4	63	–3–112	140	49

* Animals should be withdrawn from pasture on the specified dates and housed for 28 days in conditions that preclude further infection before necropsy to allow larval worms to mature and ease their recovery and identification.
[a] Either by natural challenge or by daily induced challenge.

By their very nature, these products have a greater potential to develop resistance within the parasite population than short-acting products (Dobson et al., 1996; Leathwick and Besier, 2014). It is therefore strongly encouraged/recommended that these products are as effective as possible (well above 90%).

Additional generalizations for these studies include:

1. The product tested is the one intended for the market.
2. Young animals should be used for both induced and naturally acquired infections, as the animals must remain susceptible to infection throughout the persistency period under evaluation.
3. Studies outlined in Tables 3 and 4 measure different things. In Table 3, if an anthelmintic prevents the establishment of an induced infection on Day 42, the anthelmintic has a persistent activity against infective larvae. In Table 4, Group 2, the anthelmintic would have a persistent activity of 63 days against adult worms (and possibly larval stages as well).

Once the therapeutic and prophylactic efficacy of a product has been documented by dosage determination and dosage confirmation studies, persistent reduction in FEC is tested in field studies using naturally infected animals. Field studies should be conducted at a time of the year when both transmission and parasite maturations are not interrupted (e.g. during drought/freezing conditions or when seasonal arrestment is likely). At each selected site, the animals are screened/examined for good health status and desirable coprological data (FECs and coprocultures for morphological/molecular identifications confirming the presence of targeted helminths). After appropriate blocking (e.g. age, sex, breed, FEC/FLC), the animals are allocated to treated or control groups and treatment is administered as required, followed by coprology at intervals (two to four weeks) to provide surrogate end point evidence of the therapeutic and persistent/sustained anthelmintic activity of the product for each of the claimed parasites. The aim is to determine the time of reappearance and the identity of each species when it re-establishes in the host. It is recommended that the observed efficacy is appropriately reported, e.g. "treatment enables the statistically significant reduction in FEC for n days".

The length of the study is dictated by the longest species-specific persistence period indicated on the product label. The number of studies conducted in this step of the persistent egg count reduction assessment is determined by the occurrence of the claimed parasites at the different sites. For each claimed parasite, confirmatory data must be obtained from a minimum of two different sites. The largest practicable number of animals per site should be used, consistent with the statistical aim of achieving a FECR at the expected level (>90%), with 95% confidence interval.

All other aspects of these studies are the same as for normal field studies (see above).

4. General procedures

4.1. Animal selection

For each study, animals should be in appropriate physical condition and of similar age, weight and sex. For induced (experimental) infection studies, parasite-free study animals should be inoculated with the same number of parasites from the same batch of isolated infective stages (see Section 5). For studies using animals with naturally acquired infections, the animals should not only be similar in age, weight and sex, but should also have a similar background in terms of parasite exposure and anthelmintic treatment history. For induced infections, animals should be approximately 6 months of age (some breeds of hair sheep develop immunity earlier and need to be selected at 3–4 months old) and have been raised parasite-free from birth. If this is not possible, then animals that have been rendered parasite-free (i.e., 'cleansed' of parasites) by the administration of anthelmintic prior to the induced infection may be used, provided that adequate time has elapsed to ensure that the drug is no longer active in the host. There is a danger that these animals may have already developed a degree of immunity that will interfere with subsequent induced infections. Younger animals should be used to evaluate treatment against parasites such as *Strongyloides papillosus*, which typically infect very young hosts (pre-ruminating). Animals should be infected with the helminth species for which efficacy is claimed. However, in the case of naturally acquired infections, other species and developmental life stages may be included as well.

4.2. Allocation of animals prior to study

If all study animals have similar characteristics, they should be assigned randomly to groups using a suitable statistical method, such as computer-generated allocation or with a table of random numbers. For larger group numbers (e.g. field studies, see Section 3), or if the animals are less uniform, differences between groups can be further reduced by blocking using factors such as weight, age, breed, sex, lactating status, pregnant or dry. Animals are then randomly assigned to treatment groups within the blocks. The correlation between FEC and worm burden is usually not strong for all helminths, for all ages of host or for all times of the year (Geurden et al., 2022). However, in young, recently infected ruminant livestock, which are typically selected for anthelmintic studies, the correlation is better in some parasites (McKenna, 1981; Murrell et al., 1989; Rinaldi et al., 2009). Whilst the limitations of the FEC are recognised, it is the only available indicator of worm burden and should be considered for blocking before randomly allocating animals to groups. In all ruminants, a negative FEC or FLC does not necessarily indicate absence of infection (e.g. pre-patent or arrested helminth populations or insensitive faecal egg/larvae detection methods).

4.3. Animal husbandry

As part of a study, all animals must be properly fed and watered, vaccinated according to local requirements, and handled according to good husbandry practices. In addition, all animal management and husbandry procedures must comply with national and regional animal welfare regulations and be approved by the local institutional animal welfare and ethics committees.

Experimental animals must be acclimatised to the housing facilities, the feed ration and husbandry staff for at least seven days before the start of the study. Holding facilities must be designed to provide sufficient space, shade, lighting and ventilation for each animal and fulfil the minimum requirements of national and local animal ethics committee (AEC) regulations. Individual housing or group housing should be used when testing products that could have an antiparasitic effect by simple contact between the animals (e.g. topically applied products). Individual housing/feeding can also prevent bullying and under or overfeeding

during group housing. To reduce the stress of individual housing in such situations, the animals should be able to see and hear at least one other individual. If it is necessary to demonstrate that the efficacy of a pour-on product is based solely on trans-dermal absorption and oral ingestion through grooming behaviour is not required for efficacy, this can be prevented using temporary tethering or Elizabethan collars. Husbandry staff must avoid transfer of the treatment product between groups/individual pens by taking appropriate measures such as changing personal protective equipment (overalls, boots, gloves, etc.) and using separate cleaning tools. During dosage determination and dosage confirmation studies, the animals must be housed in such a way that unplanned exposure to parasites is excluded.

The type of daily feeding should be of sufficient quality and quantity for the breed and age of the animals. The animals should be allowed to adapt to the ration at least seven days before the study begins. Feeding may be withheld 24 h prior to necropsy, but post-treatment body weights should be taken before feed withdrawal, if applicable. Water should always be available *ad libitum*, unless the treatment indication of the anthelmintic specifically advises a short period of water withdrawal. However, this is only acceptable if the animals were previously well hydrated. When in-feed or in-water products are being assessed, the amount of product ingested can be determined by monitoring individual feed and/or water intakes two days prior to treatment and during the treatment period.

In general, groups of animals may be grazed together in field studies (see Section 3) evaluating the effects of time release/bolus preparations or long-acting preparations. Co-grazing ensures that treated and untreated animals are equally exposed, even though the infection challenge to the control animals (if used) may be lower due to less pasture contamination levels caused by the treated animals. If this is not desirable or not feasible, such as with studies with topically applied anthelmintics, separate paddocks should be used for each treatment group. The quantity and quality of herbage on the separate paddocks should be as equivalent as possible and recorded in the study file. Pasture infectivity should be confirmed in the different paddocks used, using pasture larval counts or tracer animals.

4.4. Health care

The health status of experimental animals should be monitored and recorded at least daily or more frequently if required by the needs of the study or the local AEC. All supportive treatments (e.g. antibiotics, vaccines, ectoparasiticides) administered to an animal should be recorded, including indications for use, dosage, expiry dates and registration numbers. Only products that have no anthelmintic properties or antagonistic/synergistic activity on the test subject may be used.

All animals should be observed carefully for any pre-treatment abnormalities at 24 h intervals prior to treatment and the findings must be recorded. Any post-treatment observations required by the protocol due to an anticipated or unanticipated reaction to the dosing regimen will also be recorded. To prevent bias, observations must be performed by professionally trained individuals who should be unaware of the treatment groups.

5. Infection procedures

5.1. Induced infections

These can be used for dosage determination and confirmation studies against adult worms but are also generally necessary to determine and confirm efficacy against certain immature stages of parasites. Induced infections are best established in young, ruminating, helminth-naïve calves, lambs or kids in good health that have, ideally, been raised under conditions that prevent natural infection (preferably kept indoors from birth). Before infection, animals should be confirmed as free of helminth eggs by repeated coprological examinations (using the most sensitive method available) for three consecutive days and, if infected, excluded from the study.

Whilst selecting animals that have been raised helminth-free from birth is by far the preferred option, it is recognized that this is not always possible. Animals carrying a mild helminth infection can be used after the current infection has been eliminated. Choosing a treatment using a combination of short-acting broad-spectrum anthelmintics is sensible. Anthelmintics such as avermectins/milbemycins or closantel are preferably not used for this purpose due to their persistent activity. If using these products is necessary due to lack of efficacy of other drug classes, an appropriate period must elapse after treatment before the animals are enrolled in a study to avoid the persistence of the effective anthelmintic influencing the data. Fig. 1 shows the flow diagram of this regimen for small ruminants from animal selection to induced infection. Animals with induced infections should be confined under conditions that preclude further exposure to infective larvae for the duration of the study.

For each worm species included in the induced infections, the following information should be documented as described in detail in Geurden et al. (Geurden et al., 2022), with as a minimum:

1. The source location and isolation date of the parasites which should be less than 10 years earlier.
2. The anthelmintic exposure/sensitivity/susceptibility (if known).
3. Details of how the parasites have been maintained (stored in liquid nitrogen, passaged regularly in host).

5.1.1. Numbers of infective stages required for induced infections

The numbers of L_3 or metacercariae administered, as listed in Table 5, have generally resulted in sufficient infections for anthelmintic efficacy evaluation, without causing clinical disease. Some tropical breeds of ruminants may require a higher number of viable infective stages to produce adequate infection, whilst some temperate breeds may require a lower quantity. Knowledge of breed susceptibility and infectivity/pathogenicity of parasite strains may require modifications of these numbers to achieve a satisfactory balance between parasite burden and animal health. However, this guideline recommends that the study protocol specifies the minimum number of worms or larvae required to achieve adequate infection, considering the statistical, parasitological and clinical relevance of the infection level in individual control animals, rather than setting a general threshold for adequate infection in all parasite and host species (Geurden et al., 2022). Appropriate clinical observations are required to monitor all effects of infection on the host animals (see 'Preparation and administration of larval *inocula*' below).

Mixed infections with a limited number of parasite species can be achieved by combining the lower numbers of infective stages, as listed in Table 5. The exceptions are *Bunostomum* spp., for which only 500 L_3 should be used in cattle, sheep or goats, and *Gaigeria pachyscelis* and *Chabertia ovina* for which only 200 and 400 L_3, respectively, should be used in sheep and goats, when infected in combination with other helminths.

5.1.2. Hypobiotic larvae (early forth larval stage-EL_4, arrested, inhibited)

Studies testing efficacy against hypobiotic (developmentally arrested, inhibited, EL_4) larvae (e.g. *Ostertagia spp., Teladorsagia spp., Haemonchus spp.*) are best conducted with naturally acquired infections during the season(s) when arrested development typically occurs in each species. The hypobiotic nature of arrested development should be demonstrated by the recovery of L_4 in significant and broadly equal numbers in at least two animals from the group of grazing animals that have been housed worm-free for at least three weeks. This protocol ensures that all ingested larvae have had the opportunity to mature to the adult stage and that any larvae detected at *postmortem* examination are likely to be inhibited. Induced infections using 'conditioned' L_3 (Eysker, 1981; Fernández et al., 1999) can be used but the arrested development should be demonstrated in infected animals as above, prior

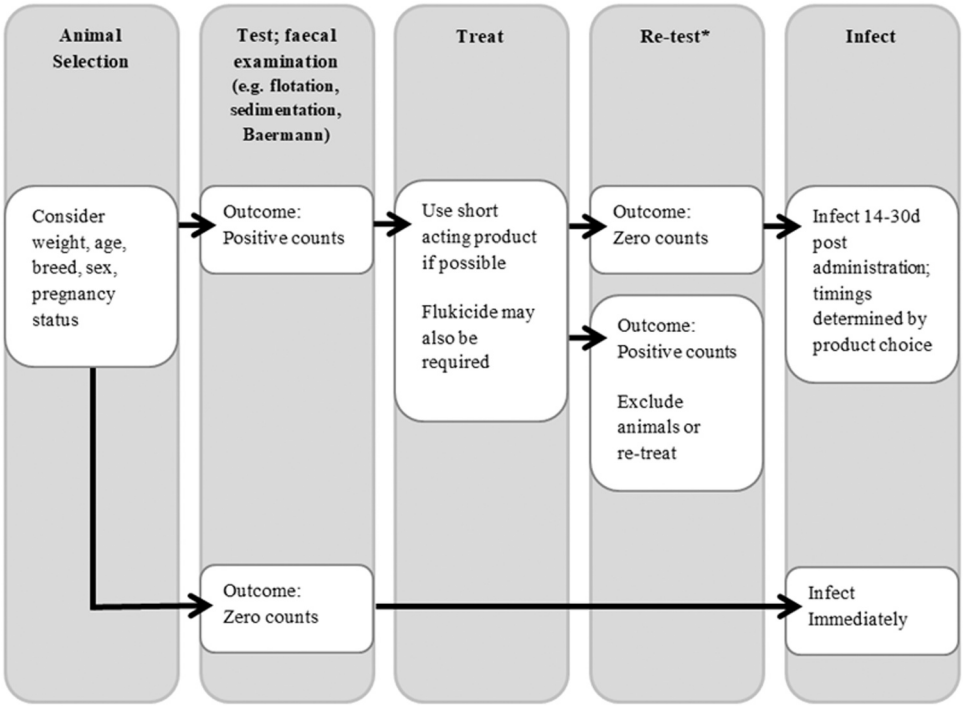

Fig. 1. A flow diagram to select small ruminants suitable for induced infection. Note: In extreme cases, a treatment regimen using a combination of anthelmintics is recommended. *Re-test timing dependent on product choice(s), i.e., if eggs/larvae are still present 14 days after this treatment, then the animals should not be considered for enrolment or require further alternative treatment.

Table 5
Numbers of infective stages (viable L_3 or metacercariae) recommended for orally induced infections in cattle, sheep and goats for anthelmintic efficacy evaluation. The lower numbers should be used where mixed infections are to be carried out. (Note, four of the nematode species are dosed using the subcutaneous/transdermal route).

Cattle	Number of larvae given
Haemonchus placei	5000–10,000
Ostertagia ostertagi	10,000–20,000
Trichostrongylus axei	10,000–15,000
Cooperia spp.	10,000–15,000
Nematodirus spp.	3000–6000
Dictyocaulus spp.	1500–3000
Bunostomum phlebotomum (topical/oral infection)	1000
Oesophagostomum radiatum/O. venulosum	1000–2500
Chabertia ovina	1000
Strongyloides papillosus (subcutaneous injection)	30,000–200,000
Fasciola hepatica (as metacercariae)	400
Sheep/goats	**Number of larvae given**
Haemonchus contortus	1500 – 4000
Teladorsagia circumcincta	5000 – 10,000
Trichostrongylus spp.	3000 – 6000
Cooperia spp.	3000 – 6000
Nematodirus spp.	3000 – 6000
Dictyocaulus spp.	1500–3000
Oesophagostomum columbianum	800
Oesophagostomum venulosum	1000
Bunostomum trigonocephalum (topical infection)	1000
Strongyloides papillosus (subcutaneous injection)	30,000–80,000
Chabertia ovina	800*
Gaigeria pachyscelis (per cutaneous infection)	400*
Fasciola hepatica (as metacercariae)	200

to commencement of the study.

5.1.3. *Preparation and administration of infective inocula*

Induced infections are usually administered orally in an aqueous suspension containing pre-determined numbers of L_3 (or other infective stage) for each parasite in the inoculum.

Clean infective larvae (separated from foreign culture material) are obtained from larval coproculture, Ministry of Agriculture, Fisheries and Food (Ministry of Agriculture, Fisheries and Food (MAFF), 1986) by various methods such as the Baermann method (e.g., MAFF, 1986) or by washing from the inner surface of a culture jar or Petri dish (Deplazes et al., 2016). Freshly harvested larvae are preferred but refrigerated stored larvae (generally less than 3–6 months old) can be used if they are active when warmed to room temperature and if the larval sheaths have not yet begun to detach from their teguments. Larvae of *S. papillosus* should not be older than 36 h to ensure their viability. Larval suspensions of most species should be stored at 4–10°C (*H. contortus* 8 ± 2°C) in tissue culture flasks (or similar vessels) in shallow water to allow sufficient aeration and at a concentration of 500–1000 larvae per ml.

After thoroughly mixing the larval suspension, at least 5–10 aliquots should be counted to estimate the number of viable L_3 per ml. If necessary, the species present can be confirmed by morphological or molecular methods (see Section 6). Thorough mixing is required at all stages of counting and dispensing. The required number of L_3 in an inoculum volume (approximately 10 ml for sheep and goats and approximately 20 ml for calves) is dispensed into syringes or narrow-necked bottles to approximately 75% of their capacity, and thoroughly mixed/agitated to keep the larvae in suspension prior to oral administration (see Table 5 for exceptions). The content of the syringe or bottle is subsequently rinsed with 5–10 ml of water and administered immediately. Attention must be given to ensure that the entire infection dose

has been swallowed before the animal is released.

Other methods can also be used for administration of infective larvae, such as containing the larvae in gelatine capsules and administering them with a balling gun (Donald et al., 1980; Van Wyk et al., 1984).

Multi-species *inocula* can be prepared by combining mono-species larval suspensions or else mixed larval cultures can be used if they are accurately identified and counted.

For hookworms, which usually infect their hosts trans-dermally (*per cutaneous*; Table 5), the larvae are either administered by subcutaneous injection (Wilkes et al., 2004), or deposited on shaved and moistened patches of skin that cannot be licked, until they have penetrated (about 20 minutes) (Yazwinski, 1988).

5.1.4. Larval moults and prepatent periods

Knowledge of the intervals between larval moults and adult patency is necessary to plan a stage-specific treatment and subsequent necropsy. The mean time required for nematodes to reach patency in a naïve host is shown in Table 6.

Studies to determine efficacies against the establishment of L_3 should be conducted using animals with induced infections. These animals should be kept under conditions which preclude any exposure to extraneous larvae. Animals should be necropsied after the larvae have completed their L_3 moult and become L_4, according to Table 6, or slaughter may be delayed until the infection is patent to facilitate recovery of the surviving worms when they reach the adult stage. This is only possible if the period of anthelmintic efficacy is shorter than the period until the next moult.

5.2. Natural infections

Natural infections are the only choice for use in field studies but can be used as well in dosage confirmation studies. The species of helminths and the relative degree and uniformity of infection prior to treatment are best determined by FEC or FLC with individual sample coprocultures for species identification by morphological means (MAFF, 1986, (Van Wyk and Mayhew, 2013) or DNA-based methods (see Section 3, Field Studies). However, it should be noted that the composition of the faecal egg population does not necessarily reflect the composition of the adult parasite population and obviously does not include pre-patent immature female worms, adult males and any arrested larval stages. In small ruminants and young cattle, a mean strongyle FEC of 300–500 and 100–150 eggs per gram (epg), respectively, would be preferable as a basis for animal selection, but a large variation in FEC is acceptable. Animals with low counts need not be excluded, but animals with FEC of 0 should be excluded. However, these are indications to be used for young (<12 months) naturally infected animals, as there is a wide range of FEC to be expected in different habitats, parasite species, host breeds and ages around the world, and local conditions must be considered.

Naturally acquired infections in cattle, sheep or goats are produced by maintaining the animals on contaminated pastures. The contaminating species of nematodes present can be determined by pasture L_3 counts, FEC, or FLC and coproculture in tracer or study animals and/or by the necropsy of tracer/sentinel animals for helminth recovery. The clinical wellbeing of experimental animals must, however, be monitored throughout the study.

Table 6
Time (days) between infection, moults (larval stages) and patency of nematodes.

Cattle	3r^{d}	4th	Patency	Authors
Haemonchus placei	1–2	14	26–28	(Bremner, 1957)
Ostertagia ostertagi	3–4	10–11	25	(Rose, 1969)
Trichostrongylus axei	4–6	10–14	21	(Douvres, 1957)
Cooperia spp.	2	8	14–17	(Keith, 1967)*
Nematodirus spathiger	<5	10–12	14–16	(Kates and Turner, 1955)
Nematodirus helvetianus	8	15	21–26	(Herlich, 1954)
Bunostomum phlebotomum	8	21–25	52–56	(Sprent, 1946)
Oesophagostomum radiatum	8–9	19	35–41	(Andrews and Maldonado, 1941)
Dictyocaulus viviparus	3	5–6	21–24	(Wood et al., 1995)
Mecistocirrus digitatus	6–9	21–28	59–82	(Fernando, 1965)
Sheep and Goats	**3r^{d}**	**4th**	**Patency**	**Authors**
Haemonchus contortus	1–5	9–11	18–21	(Veglia, 1915)
Teladorsagia circumcincta	3	7–9	17–21	(Denham, 1969)
Trichostrongylus axei	4–6	10–14	21	(Douvres, 1957)
Trichostrongylus colubriformis	3–4	8–10	21	(Mönnig, 1927)
Trichostrongylus vitrinus	3	6–9	21	(Wood et al., 1995)
Cooperia spp.	2	8	13–14	(Wood et al., 1995)
Nematodirus spathiger	<5	10–12	14–16	(Reinecke, 1973)
Nematodirus battus	7	10–12	14–16	(Reinecke, 1973)
Oesophagostomum columbianum	5–7	21	35–42	(Veglia, 1923)
Oesophagostomum venulosum	4–5	14–16	28–30	(Wood et al., 1995)
Bunostomum trigonocephalum	8	21–25	56	(Wood et al., 1995)
Gaigeria pachyscelis	7	28–35	70	(Ortlepp, 1937)
Chabertia ovina	7–8	24–25	49	(Herd, 1971)
Strongyloides papillosus	3	>6	>9	(Turner et al., 1960)
Dictyocaulus filaria	2	6–8	28–30	(Verster et al., 1971)
Marshallagia marshalli	?	?	16–21	Taylor *et al.*, 2009

* Modified by present authors.

6. Treatment

To ensure that each animal receives the correct dose of the tested anthelmintic, the following procedures help to minimize experimental errors:

6.1. Treatment schedule

Sufficient time must be allowed for the application of treatments so that the procedures can progress in an organised manner.

6.2. Record keeping

For each animal, records must be taken of the treatment dose, identification, volume, route of administration and whether the dose was delivered correctly or not.

6.3. Animal identification

Each animal must be positively identified by a unique identification number in duplicate (two ear tags, ear tag plus brand, microchip, etc.) before being allocated to a group.

6.4. Verification of weighing equipment

The scales used should be serviced regularly and checked for accuracy by an independent agency. Accuracy should be verified with calibrated weights before the first animal is weighed and after the weighing is complete. If large numbers of animals are treated or animals are being treated frequently over a prolonged period, the calibration of the equipment needs to be checked periodically during the process.

6.5. Weighing schedule

Weights of animals are taken no more than two to three days before treatment. Weighing is carried out in the same manner and at the same time of day throughout the study.

6.6. Calculation of treatment doses

The dose volume/level for each animal should be calculated based on

bodyweight and the potency given on the product label before the time of treatment. When required, doses can be rounded up to the next increment with the dosing equipment used. Administered doses must not exceed calculated doses by more than 5%. Actual doses must be recorded for each animal. Masking of the treatments should be ensured either by coding treatment containers or by having dedicated treatment staff who are then no longer involved in the study or the data analysis. The required dose is prepared, administered and any treatment failures are recorded. However, for field studies, the purpose is to test how the product performs in the field following label directions which must be followed. Here, weight brackets are often indicated (such as 20–25 kg receives x ml), with the heaviest animal in the cohort determining the weight bracket.

6.7. In-feed medication

If a formulation is administered in feed, the animals must adapt to the non-medicated feed at least 7 days before treatment to check palatability and intake by the individual/group. The amount of feed consumed daily by each individual animal should be determined and recorded from two days before treatment and until the end of the treatment period. Samples of the medicated feed should be collected, appropriately stored, and analysed prior to the known in-feed stability period of the product, to confirm the drug concentration.

6.8. Injected medication

For intra-muscular and sub-cutaneous administration, the injection sites should be clearly defined and recorded for each treatment in a manner that allows any lesions or morphological changes related to the tested drug formulation/administration to be identified after treatment. In some cases, shaving the injection site before treatment may facilitate these observations. Animals with pre-study treatment site anomalies should not be used.

6.9. Oral administration

For oral suspensions, a mixing or stirring protocol may be required to produce a homogeneous suspension of the active ingredient immediately prior to administration but as far as possible, avoiding bubbles that could affect the correct dose offered to the animals. Aliquots of the batch of product to be administered are stored appropriately for subsequent analysis. Some researchers prefer to use a stomach tube for dosage administration due to concerns about oesophageal groove closure.

6.10. Drinking water medication

If treatment is administered via drinking water, the amount of water consumed before and during the treatment phase of the study is recorded daily with due allowance given for evaporation, especially in dry or hot areas of the world. Samples of the medicated water must be taken within the known stability period of the product and prior to treatment, stored appropriately and analysed to confirm the drug concentration.

6.11. Topical administration

When using topically applied products outdoors, such as in field studies/natural infections, the treatment should not begin on days when rain is expected. If treatments are programmed under such conditions and no delay is acceptable, the animals should be kept under cover for 24 h. If this is not possible, rainfall must be recorded during treatment and for 24 h post-treatment, as well as temperature, humidity and duration of sunshine. This will help to identify any potential negative effects on efficacy. Any soiling of the treatment site should be cleaned and dried. Animals with active skin lesions at the treatment site or other skin problems must be avoided when testing topical formulations. Animals should not be enrolled in studies if lesions caused by ectoparasites are present on or near the topical treatment or injection sites. Individual pens must be considered to avoid drug transfer between animals and Elizabethan collars, or temporary tethering may be used to avoid ingestion of drug by allo-grooming (if this behaviour is undesirable for the study).

6.12. Treatment errors

Investigators must record any problem with the administration of the treatment, such as spillage, under-dosing, accidental over-dosing, incorrect injection or regurgitation of the product after drenching.

6.13. Induced infections

The following treatment schedule is designed for studies involving induced infections.

a. For adult helminths, treatment should not be administered before patency (see Table 5).
b. For $L_{3\ and}$ $L_{4,}$ treatment should be carried out according to the schedule in Table 6. After treatment, parasites remaining in both treated and control animals should be recovered at necropsy before the scheduled moult to the next stage. However, this can be technically difficult to achieve for the treatment to work and for the killed larvae to be dislodged from their predilection site. Therefore, necropsy may be delayed allowing worms to reach the adult stage and facilitate their recovery and identification, but only if the test product is of short duration relative to the stage tested (less than the normal interval between moults, see Table 6). If this is not the case, the actual stage killed cannot be accurately determined.

6.14. Post-treatment observations

After treatment, all animals must be observed for at least 4 hours and daily thereafter to record any undesirable side-effects of the treatment. Observations include lesions at the site of treatment, any change in the skin or hair of the animals, nervous signs, reduced food or water intake, abnormal behaviours and any other abnormalities. If adverse reactions occur, the animals should be observed at intervals dictated by the nature and severity of the clinical signs until they are normal, or the animals are withdrawn from the study on welfare grounds. All animals that die or have to be euthanized before scheduled necropsy should undergo *post-mortem* examination by a suitably qualified person and the cause of death determined.

6.15. Record of treatment data

The studies should be conducted according to the standards of GCP (good clinical practice) and/or GLP (good laboratory practice). Data must include all individually numbered animals with their body weight and treatment dosage, including any treatment failures, the identification numbers of the test products, the route of administration, identification of animals exhibiting abnormal behavioural changes after treatment and any treatment site lesions. The names of the individual(s) treating and checking animals, preparing the doses, observing behaviour and performing any necessary veterinary interventions required should also be recorded.

7. Necropsy procedures, parasite recoveries and identification

7.1. Scheduling

When the necropsies of all animals cannot be completed in a single day, an equal number of animals from each experimental group should be processed each day so that all study animals are necropsied within

2–4 days.

7.2. Necropsy technique

The identification of each animal must be carefully checked before and after necropsy. On removal, all organs to be examined must be labelled and clearly identified. Animals must be handled and euthanised in a humane manner in accordance with national animal welfare regulations and guidelines of the country in which the study is conducted.

7.3. Organ collection and processing

The processing of organs for the recovery and identification of helminths should be carried out by experienced and suitably qualified personnel. The wide range of helminth species found in ruminants leads to different host-parasite interactions around the world. Each laboratory will have developed their own processing methods to take account of local conditions and it is not our intention to suggest a completely standardised approach. However, the protocols used must be carefully applied and recorded throughout the study for all animals/samples, clearly described and reproducible. The method used to collect aliquots must comply with parasitological and statistical requirements. Some common methods that have been used successfully in many laboratories over the years are presented below.

7.4. Gastro-intestinal tract

Before the gastro-intestinal tract is removed, double ligatures or clamps are placed at the omasal and pyloric ends of the abomasum and on the ileocecal junction. The abomasum, small and large intestines are separated, and excess fat and mesenteric attachments are removed.

7.5. Abomasal contents

The abomasum is opened longitudinally along the greater curvature and its contents thoroughly washed under a slow stream of water into a graduated bucket or rinsed in a bucket containing water or physiological saline (PS; 0.85% NaCl). The abomasal surface and folds are then washed thoroughly and the washings added to the contents. The volume of the washed contents should be made up to a known volume (generally 4 L for most ruminants). In some cases, a larger volume of water may be required to properly collect and wash the contents, thus the volume of collected contents/washings must be recorded. After thorough mixing, at least two 5% aliquots (larger aliquots are perfectly acceptable) are withdrawn from the bucket, sieved separately and mixed with sufficient fixative such as iodine solution (40%), 70% ethanol or 10% formalin (being aware of the potential health hazards and risks associated with this chemical)) for preservation until subsequent examination. Some laboratories prefer to fix the aliquots and carry out the sieving later. The remainder of the bucket (either sieved or un-sieved) may be fixed and stored until the aliquots have been successfully processed. Some laboratories prefer to sieve and fix the entire contents at the time of *post-mortem* and only aliquot them shortly before examination. Some laboratories prefer to rapidly freeze the organs/organ contents on the day of collection and process later to reduce the workload on necropsy days Ciordia et al. (Ciordia et al., 1957). The choice of sieve size depends on the species of the parasite involved as well as the stage of parasite under investigation. Adult ruminant nematodes are readily retained on a 150 μm sieve whilst studies specifically targeted at larval stages require a 38 μm sieve. Studies where larval and adult stages are both likely to be present require at least both of the above-mentioned sieve sizes (some laboratories prefer to run the samples through a series of sieves to facilitate the examination of each individual sample). For possible DNA work, fresh living subsamples or isolated helminths can be preserved in ethanol at a final concentration of 70%.

7.6. Abomasal mucosa

The recovery of tissue-dwelling larval stages requires the abomasal mucosa to be processed by one of two methods:

7.6.1. Saline incubation method

After processing the contents, the abomasum is soaked, mucosal surface down, in physiological saline (PS) at 37–40°C for a minimum of 4 h (Williams et al., 1979; Williams et al., 1981). After soaking, the abomasum is removed from the PS and each fold is squeezed between the fingers into the PS to remove the mucus layer and any retained adherent worms. During this procedure nearly all the larvae will have migrated from the mucosa into the PS and can be recovered intact. Once thoroughly cleansed (washing with manual abrasion), the abomasum can be disposed of appropriately. The collected soak/washings are made up to a standard volume (2 or 4 L), thoroughly mixed and two 5% aliquots are collected, sieved through a 38 μm sieve and fixed for later identification and counting (or are fixed first and sieved later). The remainder of the contents can be fixed and stored until the aliquots have been successfully processed and the contents are no longer required. This method cannot be used if the abomasum has been previously frozen.

7.6.2. Pepsin + hydrochloric acid (HCl) or HCl digestion method

The mucosal surface is scraped off from the abomasum using a knife or a glass microscope slide, weighed and added to the digestive solution, which may be either 1% pepsin in 3% hydrochloric acid (HCl) or just 3% HCl. The volume, by weight, of this solution should be at least three times the weight of the mucosa. The mucosal material is digested in the digestive solution in a water bath at 37–40°C for no longer than 4–6 hours. Both the temperature and duration of digestion must be controlled since overheating and/or prolonged digestion will destroy nematodes, so periodic examination during digestion is advised to determine the end point. When carefully controlled, this procedure may recover about 10% more larvae than the saline incubation method described above (Downey, 1981). The digestion may then be poured through a 38 μm sieve and the residue examined for parasites or diluted with tap water to 2–4 L (the exact volume recorded), the suspension thoroughly mixed and two 5% aliquots (or larger) removed and fixed for later examination. If large numbers of larvae are present, a sub-aliquot of appropriate size must be taken. The aim is to isolate and identify at least 200–300 worms per sample.

7.7. Small intestine contents

The entire length of the small intestine is opened into a large container to collect the contents. The opened intestine is rinsed twice in water or PS and the contents squeezed out by pulling the gut through the tightly clenched fingers of one hand. All the contents and washings are added to the same container. The contents are made up to a known volume (usually 4 L), depending upon the size of animal but consistent for all animals necropsied. The suspension is thoroughly mixed, and two 5% aliquots are removed, sieved and fixed (or fixed and sieved) for later examination. The remainder of the container may be fixed and stored until the aliquots have been successfully processed.

For the recovery of tissue-dwelling larval stages, the small intestinal mucosa needs to be processed as follows:

7.7.1. Saline incubation

This technique is useful for the recovery of both larval stages and attached *Bunostomum* spp. (Yazwinski, 1988), histotrophic hookworms, scoleces of *Moniezia* spp. and villus-retained nematodes such as *Cooperia* spp., *Nematodirus* spp., *Trichostrongylus colubriformis* and *Strongyloides papillosus*. After the process described above, the small intestine is soaked in PS or tap water at 37–40°C for 4–6 h. The tissue is again run through the fingers into the saline to remove the mucus and any

adherent worms, and the volume of the collection is made up to a minimum of 2 L and 4 L for small ruminants and cattle, respectively (the exact volume is recorded). The suspension is thoroughly mixed and two 5% (or greater) aliquots are removed, sieved through a 150/38 μm sieve (as appropriate) and preserved for later examination. The aliquots can be fixed immediately and sieved later for examination. The remainder of the suspension can be fixed and stored until the aliquots have been processed.

7.8. Large intestine contents

The contents of the large intestine and caecum are collected and processed in the same way as the small intestine. However, a larger sieve size can be used to retain adult worms (200 μm – 300 μm) and a 50 μm – 65 μm is sufficient to retain larval stages. After washing and content collection, a further step may be required to recover the larval stages of *Chabertia* spp., *Oesophagostomum* spp. or *Trichuris* spp. as well as attached adult stages. The intestine is soaked in water or PS at room temperature for 4–6 h, washed and examined for attached nematodes, which are collected, identified and counted. The large intestine is then discarded. The total soak fluid is washed through a 150 μm – 50 μm sieve (as required) and the residue examined immediately or preserved for later examination. If species of different sizes or larval stages are present, it is often helpful to use a series of sieves and count the collected residues separately.

7.9. Lungs

The lungs with trachea attached are removed, ensuring that damage to the lobes is minimized. The trachea and bronchi are incised lengthwise with appropriately sized scissors and the main and lateral bronchioles are opened systematically. Adult worms are removed and stored in fixative for later enumeration. The fixation reduces the likelihood of worm entanglements, which complicate enumeration. The recovering of worms is increased by soaking the 'opened lungs' in PS for a minimum of 4 h at room temperature, followed by washing in tap water. The smaller lungworm, *Protostrongylus rufescens* can be processed in a similar way, as its brown colour enhances visibility. For examination of immature stages, the lungs are sliced into small pieces and incubated at 37°C in PS for a minimum of 4 h. After incubation, the pieces of lungs are rinsed, and the soak liquid passed through a 38 μm sieve to collect the worms and their remnants. The entire residue from each animal can be stored in fixative for later examination.

A perfusion procedure is an alternative method to those mentioned above (Oakley, 1980). In this case, the lungs, trachea and heart are removed intact. Tap water is perfused through the entire lung tissue at normal tap water pressure (1–1.5 Bar) via a cannula connected to the pulmonary artery. The water bursts into the alveoli and flushes most adult and larval stages of worms out through the trachea. The flushing water is collected in a large bucket (20 L) and subsequently passed through a 38 μm sieve. To prevent excessive foaming during the sieving process, 85 g NaCl and 5–10 ml octanol can be added to the bucket. The worms in the residue after sieving are counted and identified according to species and stage of development. This method is especially useful for recovering mature and immature stages of *Dictyocaulus* spp. but for inhibited stages a combination of perfusion with the Baermann technique is required (Eysker et al., 1990).

7.10. Liver

Before the liver is removed from the carcass, the small intestine is ligated or clamped 50 cm either side of the bile duct entry point to prevent the escape of flukes (if the small intestine is also processed for nematode recovery, this ligation is omitted and any flukes in the small intestine are recovered with the nematodes). The gall bladder is removed, and the bile is stored to be examined later for flukes and/or fluke eggs. Once the liver is removed, all accessible bile ducts are opened using fine point scissors and the flukes are physically recovered with forceps. This recovery is aided by applying pressure to the areas around the deeper uncut ducts to squeeze out the flukes. The remaining liver sections are cut into one cm thick slices and pressure is applied to squeeze flukes out of the smaller bile ducts. The slices are soaked overnight in PS and then washed in tap water. The washings are passed through a 300 μm sieve and the entire residue on the sieve examined for flukes. Total and partial flukes can be counted. Flukes will have been cut into pieces during the processing so it will usually be necessary to identify, count and record heads or tails separately. If no adult flukes are detectable, the presence of fluke eggs in the bile is an indicator of undetected adult fluke infection at necropsy. The bile can be washed through a 52 μm sieve and the residue on the sieve examined for the presence of trematode eggs.

7.11. Rumen contents

Before removing the rumen/reticulum, ligatures or clamps are placed at the oesophagus and reticulum. The rumen is opened along the greater curvature and the contents carefully removed. Adult flukes are found adhered to the rumen wall, in the reticular groove and caudal pillar (with a few in the reticulum, ventral and dorsal sac) and can be manually removed, identified and counted. Species identification of rumen flukes is not a straightforward process and requires specimens to be examined after histological preparation or preferably identified by molecular means (Mitchell et al., 2021). Immature flukes can spend up to six weeks in the small intestine, mainly in the duodenum and abomasum (e.g. as with *Paramphistomum microbothrium,* (Horak, 1967)). Therefore, these organs must also be examined if a study aims to measure efficacy against immature stages. Immature flukes are mainly found attached to and in the mucosa but can also be found unattached in the ingesta of these organs. To obtain them, the contents of the organs should be collected and washed through sieves with a mesh size of 200 μm and 60 μm. The mucosa of the small intestine and abomasum is thoroughly washed, scraped off and all the collected material sieved with the organ contents. The residue in the sieves is transferred into a container and fixed for later examination ((Horak, 1967; Rolfe and Boray, 1987)).

7.12. Laboratory procedures

It is recommended that samples are coded in some way so that the investigators collecting the data can recognize the treatment group. Aliquots of all contents, soaks and digests collected as described above are appropriately examined for all parasites. If parasites are numerous (e.g. greater than 300 worms per aliquot), accurately measured subsamples from well-stirred residues can be used.

Where duplicate sub-samples were taken at necropsy, one of these samples serves as a back-up in case of an accident. If low parasite burdens are found in control animals (<200), the analysed sample size can be increased. The final total volume of all samples and the number and size of aliquots taken are recorded. In a mixed infection study, if one species has a high worm burden and another a low worm burden, then different proportions of the aliquot may have to be counted for each species.

7.12.1. Recovering, counting and identifying GI nematodes

The aliquots from the abomasum or small intestine contents should be washed through a series of sieves with decreasing aperture, from 1000 μm to remove any gross debris followed by 150 μm to 38 μm, so that all forms are readily isolated and counted with a minimum of debris. However, if the samples are relatively clean, then only the smaller of the sieves needs to be used. The residues are transferred into wide-mouthed jars and fixed for later examination.

Aliquots from soaks and digestions should be washed through sieves

of 38 μm aperture to capture any larval stages.

Counting procedures should be the same for all samples, with the careful recording of the numbers, species and stages of nematodes in each aliquot from each animal. To stain the nematodes, a few drops of 45% iodine solution or helminthological iodine (50 g iodine, 250 g potassium iodide in 500 ml distilled water) can be added to a sample. Subsequently, the background is cleared with 5% sodium thiosulphate solution immediately prior to examination, so that the stained parasites can be seen more clearly. The samples are poured into shallow dishes (e.g. Petri dishes) and the worms identified and counted using a stereomicroscope (10–40X magnification) with trans-illuminating light. With experience, parasites can be identified directly with a stereomicroscope during the counting procedure. However, it is not always possible to identify the worm species/larval stages in this way, so it is often necessary to mount specimens on slides and carry out the identification using a compound microscope. Nematodes are identified using the characteristics described by MAFF (1986), Clark et al. (1971), Reinecke (1973), Clark and Turton (1973), Thomas and Probert (1993), Ueno and Gonçalves (1998), Barth and Visser (1991) or other publications including the descriptions of specimens. Several species, including *Ostertagia* spp., *Teladorsagia* spp. and *Cooperia* spp., can show distinct morphological variations (morphotypes) within their male populations. During processing, these are often identified and counted based on their morphological groups. However, for efficacy calculations, the morphological groups within a species are considered synonymous. For female species such as those within the genera *Ostertagia/Teladorsagia* and those within the genus *Trichostrongylus*, which cannot easily be separated into individual species, it is acceptable to divide the populations within each genus in the same proportion as the males of the species that can be identified.

For most anthelmintic efficacy studies, morphological identification is still likely to remain the method of choice. Importantly, the identification needs to be carried out by experienced staff using validated published descriptions.

Most trichostrongyle eggs cannot be accurately identified morphologically and must be cultured to L_3 for identification. Infective stages of helminths such as L_3, have been identified morphologically using the characteristics published by MAFF (1986), Van Wyk et al. (2004) and others. This has been a major part of species identification after coproculture to determine the proportion of species and/or genera present in a given sample and is often a laborious process that can only be carried out by experienced individuals. However, advances in molecular technology have made it possible to perform the species identification of eggs, L_3 and adult stages and these techniques will be used more in future (see Section 3).

7.12.2. Material for molecular analysis

When collecting material for species identification using molecular techniques (see 3.3), it is important to consider how and when it should be collected, stored and analysed. Note that conventional fixatives are not suitable for molecular analysis. Therefore, separate samples must be taken from the contents or samples before fixatives are added. Storage of worms/larvae at approximately −18°C or in 70% ethanol (v/v) are suitable for downstream molecular work.

7.12.3. Specific evaluation studies for lungworms

7.12.3.1. Dictyocaulus spp. Two commonly occurring ruminant lungworms are *Dictyocaulus viviparus* in cattle and *Dictyocaulus filaria* in small ruminants. Dosage determination and dosage confirmation studies targeting either of these two nematodes are often conducted at the same time and in the same studies as those targeting GI nematodes, using either natural or induced infections. In field study evaluations, natural infections of cattle, sheep or goats with *Dictyocaulus* spp. are monitored using the Baermann procedure (Rode and Jorgensen, 1989; Eysker, 1997), in which harvested larvae are counted and identified microscopically. It should be noted that there is little correlation between FLC and adult nematode or larval populations, so care should be taken when selecting suitable animals for the study. In practice, only animals with positive FLC (however low in number) are used.

For induced infections (dosage determination and dosage confirmation studies) larvae are harvested from the faeces of donor animal and matured to the infective stage (MAFF, 1986). Generally, 1500–3000 L3 per animal are administered or 25 L_3 per kg body weight. However, animals of similar weight should be used and the same number of L3 should be administered to all experimental animals. Lungworm larvae can be co-administered with gastro-intestinal (GI) nematode larvae. In susceptible cattle, the third and fourth moults of *D. viviparus* occur 3 days and 5–6 days post-infection, respectively, with mature adults present 21–24 days post-infection (see Table 6). In sheep and goats, third and fourth moults of *D. filaria* occur 2 days and 6–8 days post-infection, respectively, with a pre-patent period of 28–30 days (see Table 6).

Inducing lungworm infections can be difficult as the host immune response is generally very pronounced and effective, especially in animals that have been previously infected. This can be overcome by an appropriately portioned number of L3 given daily as a short-term trickle infection (e.g. twice daily for 2–3 days). It should be noted that animals infected with other nematodes that migrate through or reside in the lungs may also develop resistance against *Dictyocaulus* spp. (Horak, 1971).

The anthelmintic efficacy of *Dictyocaulus* spp. is generally determined against adult parasites which can be targeted 28 days after all L3 have been administered (induced infections), or after naturally infected animals have been removed from challenge for at least 28 days. The timing of necropsy will depend on the anthelmintic pharmacokinetics, clearance time for killed parasites, and factors pertinent to the inclusion of other targeted helminths in the study.

There is evidence that the development of larval stages can become arrested under certain conditions (Laabs et al., 2012). Determination of anthelmintic activity against arrested larval stages of *Dictyocaulus* spp. is therefore useful, although *postmortem* recovery of these stages can be difficult. A digestion technique such as the one used by Eysker et al. (1990). or (Rehbein and Visser, 2002) for *Muellerius capillaris* is recommended. Methods for worm recovery at necropsy can be found in the Necropsy Section (see Section 7.9.).

7.12.3.2. Studies with other small lungworms. Due to the indirect life cycles of *Protostrongylus rufescens, Muellerius* spp., and *Cystocaulus* spp., efficacy studies with these species are generally carried out in naturally infected animals. In addition, the detection and quantification of all these lungworm parasites at necropsy is very difficult, but it can be enhanced using a pepsin digestion technique (Rehbein and Visser, 2002)

Given the above, these parasites, in addition to other parasitic helminths of similar, minor relevance (discussed elsewhere), will eventually be treated by prescription after anthelmintics for similar genera of parasites have been evaluated and approved. Although these parasites are not listed in this guideline, it is encouraged that any anthelmintic treatment prescribed is evaluated for efficacy and suitably reported (e.g. case reports, scientific meeting presentations, etc.).

8. Specific guidance on helminth groups other than nematodes

8.1. Fasciola hepatica

In general, the same number of dosage determination, dosage confirmation and field studies should be planned with the same basic designs as for nematodes but adapted to the studies with *F. hepatica*.

Dosage determination and dosage confirmation studies are controlled studies, usually conducted with induced infections in cattle, sheep and goats that have had no history of prior infections. This is best

done by securing study animals that have not grazed or animals from locations where there has been no fluke infection. The parasite-free status should be confirmed by 3 contiguous daily negative FEC and a serological/copro-antigen ELISA (Rojas et al., 2014). Due to the length of time that juvenile flukes remain in the liver parenchyma, FEC must be carried out at least 12 weeks since removal from potentially infective pasture. The efficacy of flukicides should be determined for both immature and mature infections, as these have shown dissimilar levels of susceptibility to drugs in the past (Forbes et al., 2015). To provide accurate information on the efficacy of flukicides relative to fluke age, the following post-infection times for anthelmintic treatment are advised:

8.1.1. Early immature stages

Treatment at 1–4 weeks post-infection, flukes will be migrating in the liver parenchyma.

8.1.2. Late immature stages

Treatment at 6–8 weeks post-infection, flukes are still immature, but localized in the hepatic bile ducts.

8.1.3. Mature flukes

Treatment at 12–14 weeks post-infection. All forms are in the bile ducts and gall bladder.

Oral infection with *F. hepatica* metacercariae at approximately 400 per animal for cattle and approximately 200 for sheep or goats will result in adequate adult fluke burdens for a meaningful anthelmintic treatment evaluation. Generally, patency is reached earlier in small ruminants than in cattle. The viability of metacercariae in the inocula should be assessed prior to oral administration. Microscopic inspection of infective metacercariae after decortication (with sodium hypochlorite or mechanically) should reveal the distinct internal morphology indicative of viable specimens (Boray, 1969). Alternatively, *in vitro* egg-viability assays can be used (Chryssafidis et al., 2015).

For the recovery of flukes at necropsy, see Section 6.

In dosage determination and dosage confirmation studies, efficacy is determined by comparing the number of flukes in treated animals with those in untreated control animals.

In field studies, anthelmintic activity is determined by comparing the FEC from treated animals shortly before or at the time of treatment and then again at least 3 weeks later. Alternatively, an untreated control group could be included to provide better evidence of actual efficacy and to compare any adverse reactions under field conditions (see Section 3). Quantifying trematode eggs in faeces is less accurate than for nematode eggs because fluke egg expulsion is sporadic and the dense flotation solutions required to isolate the eggs (e.g. zinc sulphate, SG 1.45) produce more debris in the samples observed under the microscope. Due to the sporadic shedding of eggs, it is recommended to collect faecal samples on three consecutive days. Sedimentation methods can be more accurate and sensitive than flotation ones for trematode eggs (Conceicao et al., 2002). For field studies, flocks or herds with a history of fluke infections are best used, at a time of year when recent fluke infections have reached patency. Samples for FEC should be taken before the study is conducted. However, the correlation between egg count and worm burden is not necessarily strong. Animals with positive egg counts should be first blocked by body weight, age, breed and sex, and then randomly distributed to study groups.

8.2. Fasciola gigantica and Fascioloides magna

Because *F. magna* has a different parasitic life cycle in cattle and small ruminants compared to *F. hepatica*, it is difficult to carry out studies using induced infections. The greater significance of *F. gigantica* in some parts of the world may warrant conducting dosage confirmation studies. In these cases, it is recommended to follow the guidance for *F. hepatica*. In addition, it is recommended that studies evaluating field efficacy against *F. magna* and *F. gigantica* are reported via publications and scientific presentations.

8.3. Dicrocoelium spp

Induced infections with *Dicrocoelium* spp. are difficult to achieve, therefore efficacy studies are usually carried out with naturally infected animals. Conventional faecal sedimentation or egg counting techniques using a high-density flotation solution, e.g. saturated $ZnCl_2$ or $ZnSO_4$ solutions (SG 1.45) can be used to select infected animals. The correlation between egg count and worm burden is not necessarily strong; therefore, animals with positive egg counts should be distributed randomly to study groups after they have been blocked by body weight, age, breed and sex. The animals should be kept under conditions that prevent natural re-infection with *Dicrocoelium* spp. from shortly before treatment and throughout the study.

Necropsies are carried out 14 days after treatment to identify and count the worms. Immediately after slaughter, the first 3–4 m section of the small intestine is ligated, as parasites may be present in that part. The number of *Dicrocoelium* in the liver can be determined by sequentially cutting the entire organ into slices about 1 cm thick while applying downward pressure onto the remaining uncut remnant of the liver to squeeze the flukes out of the bile ducts. In addition, the slices are massaged vigorously in warm physiological saline to recover further flukes.

Liquid perfusion of the bile ducts (under pressure) has been described as an alternative time-saving method with a recovery rate of about 90% of the worm burden (Wolff et al., 1969). Sieving techniques, as described for the recovery of nematodes from the abomasum may be used for collecting *Dicrocoelium* from the small intestine.

In field studies, FEC, although highly variable, is the only practical method for monitoring anthelmintic activity in larger groups of sheep, goats and cattle. Egg counts are conducted at least three times during the week before treatment and 7, 14 and 30 days after treatment. However, it should be noted that longer observation periods may be necessary for certain products, as the reproductive organs of the flukes may be only temporarily damaged and interrupted egg laying activity may be restored.

8.4. Paramphistomatids

To evaluate efficacy against *Calicophorum cervi* or *Paramphistomum daubneyi*, a controlled study should be conducted with naturally infected animals or induced infections of 20,000 metacercariae per sheep/goat or 50,000 per calf. For immature flukes, treatment should be administered 7–14 days after infection while the young flukes are still in the small intestine. The necropsy should be carried out 21 days post treatment, before migration to the rumen takes place. For adult flukes, treatment is recommended at 2.5 months after infection, and the necropsy, one week after treatment.

8.5. Studies with adult tapeworms

8.5.1. Moniezia spp

Infections with *Moniezia* spp. are often the predominant tapeworm infections (*M. benedeni* in cattle and *M. expansa* in small ruminants). Only animals with natural tapeworm infections are used for the evaluation of anthelmintics, as it is extremely difficult to use artificially induced infections due to the heteroxenous life cycle of these helminths. Animals with naturally acquired infections are identified by the detection of characteristic gravid segments or eggs in faeces that have been appropriately processed for flotation, isolation, concentration and identification of the eggs. Once a suitable number of animals has been identified, the infected animals are randomly allocated to treatment groups as required.

For controlled studies (dosage determination and dosage

confirmation), the same requirements apply as described above for the conduct of controlled studies. The study animals are kept away from potential sources of infection for at least 14 days, randomly allocated to groups and then treated. At least 12 days should elapse between treatment and necropsy to allow the drug to complete its effects and to allow time for the development of new proglottids from the scoleces that have not been removed by drug action. It should be noted that there is a tendency for self-cure to occur when infected animals are brought to the study site and subjected to a change of diet and husbandry (Reinecke, 1980).

At necropsy, personnel experienced in the recovery of cestodes must conduct the helminth retrievals. Initially, the entire ligated small intestine is removed and opened lengthwise. The totality of the content is collected with a light wash (Collection 1). During this process, care must be exercised not to remove any attached scolexes, as these are much easier to isolate and quantify from the soak fluids described below (Collection 2). Also, during this process any non-attached strobila or segments in the ingesta are retrieved with forceps and preserved for later stereoscopic viewing and scolex/neck identification. The predetermined aliquot from Collection 1 is sieved through a 150 μm sieve and the residue suitably preserved. The intestine is soaked in tap water at room temperature for 4 h. It is then removed and scrapped off lengthwise, through the fingers of a clenched fist, and all fluids and intestinal slurry/mucus are collected (Collection 2). Collection 2 is then gently washed with a warm water spray over a 150 μm sieve. All sieve residues are collected altogether and fixed before sieving again to remove the fixative and analyse the residues for scolexes or necks with final proglottid identification. Isolation of tapeworm strobila can be aided by viewing the white tapeworms in a black tray. The counts of scoleces and necks from Collections 1 and 2 are combined and the total *Moniezia* spp. count for an animal is the scolex or neck, whichever is greater. For a controlled study to be reliable in contrasting control and treated animal burdens and predict levels of drug efficacy, a minimum of six control animals must be infected at necropsy.

8.5.2. Other tapeworms

Whilst ruminants are infected with other cestodes such as *Thysanosoma actinoides* and *Thysaniezia giardia,* their incidence and prevalence are not sufficient to warrant all steps of anthelmintic evaluation. Rather, it is assumed that commercially available cestocides against *Moniezia* spp. will be prescribed for these more infrequent parasites and the results should be published and/or presented at peer-attended meetings.

CRediT authorship contribution statement

T. Yazwinski: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **David Burden:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **R B Besier:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **D J Bartley:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **T P Elliott:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **E. Claerebout:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **S. Rehbein:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **J. Höglund:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **J A Van Wyk:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **J F.J. Torres-Acosta:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this guideline.

Acknowledgements

The authors would like to thank Alessandro Amarante, Joan Burke, Silvina Fernández, William Pomroy and Nick Sangster for reviewing the manuscript, and Thomas Geurden for the helpful comments during the preparation of this paper.

References

Andrews, J.S., Maldonado, J.F., 1941. The life history of *Oesophagostomum radiatum,* the common nodular worm of cattle. Res. Bull. P. Rico Univ. Agric. Exp. Stn. (No 2).
Avramenko, R.W., Bras, A., Redman, E.M., Woodbury, M.R., Wagner, B., Shury, T., Liccioli, S., Windeyer, M.C., Gilleard, J.S., 2018. High species diversity of trichostrongyle parasite communities within and between Western Canadian commercial and conservation bison herds revealed by nemabiome metabarcoding. Parasit. Vectors 11, 299.
Avramenko, R.W., Redman, E.M., Lewis, R., Yazwinski, T.A., Wasmuth, J.D., Gilleard, J. S., 2015. Exploring the gastrointestinal "nemabiome": deep amplicon sequencing to quantify the species composition of parasitic nematode communities. PLoS One 10, 143559.
Avramenko, R.W., Redman, E.M., Lewis, R., Bichuette, M.A., Palmeira, B.M., Yazwinski, T.A., Gilleard, J.S., 2017. The use of nemabiome metabarcoding to explore gastro-intestinal nematode species diversity and anthelmintic treatment effectiveness in beef calves. Int. J. Parasitol. 47, 893–902.
Baltrušis, P., Halvarsson, P., Höglund, J., 2019. Molecular detection of two major gastrointestinal parasite genera in cattle using a novel droplet digital PCR approach. Parasitol. Res. 118, 2901–2907.
Barth, D., Visser, M., 1991. Magen-Darmnematoden des Rindes: Diagnostischer Atlas. Ferdinand Enke Verlag, Stuttgart, p. 104.
Bisset, S.A., Knight, J.S., Bouchet, C.L.G., 2014. A multiplex PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures and/or pasture samples. Vet. Parasitol. 200, 117–127.
Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv. Parasitol. 7, 95–209.
Bremner, K.C., 1957. The parasitic life cycle of *Haemonchus placei.* Aust. J., Zool. 4, 146–151.
Chryssafidis, A.L., Fu, Y., De Waal, T., Mulcahy, G., 2015. Standardisation of egg-viability assays for *Fasciola hepatica* and *Calicophoron daubneyi*: a tool for evaluating new technologies of parasite control. Vet. Parasitol. 210, 25–31.
Ciordia, H., Vegors, H.H., Bizzell, W.E., 1957. Freezing procedures for greater flexibility in application of the digestive method for *post mortem* recovery of cattle nematodes. J. Parasitol. 43, 532–534.
Clark, C.J., Tucker, A.M., Turton, J.A., 1971. Sampling technique for estimating roundworm burdens of sheep and cattle. Exp. Parasitol. 30, 181–186.
Clark, C.J., Turton, J.A., 1973. Estimating roundworm burden and group sizes in anthelmintic trials with sheep and cattle. Exp. Parasitol. 34, 69–75.
Conceicao, M.A., Durão, R.M., Costa, I.H., Correiada da Costa, J.M., 2002. Evaluation of a simple sedimentation method (modified McMaster) for diagnosis of bovine fascioliosis. Vet. Parasitol. 105, 337–343.
De Seram, E.K., Redman, E.M., Wills, F.K., Queiros, C., Campbell, J.R., Waldner, C.L., Parker, S.E., Avramenko, R.W., Gilleard, J.S., Uehlinger, F.D., 2022. Regional heterogeneity and unexpectedly high abundance of *Cooperia punctata* in beef cattle at a northern latitude revealed by ITS-2 rDNA nemabiome metabarcoding. Parasit. Vectors 15, 17.
Denham, D.A., 1969. The development of *Ostertagia circumcincta* in lambs. J. Helminthol. 43, 299–310.
Denwood, M.J., Kaplan, R.M., McKendrick, I.J., Thamsborg, S.M., Nielsen, M.K., Levecke, B., 2023. A statistical framework for calculating prospective sample sizes and classifying efficacy results for faecal egg count reduction tests in ruminants, horses and swine. Vet. Parasitol. 314, 109867.
Deplazes, P., Eckert, J., Mathis, A., Samson-Himmelstjerna, G., Zahner, A., 2016. Parasitology in Veterinary Medicine, 653. Wageningen Academic Publishers, Wageningen.
Dobson, R.J., Le Jambre, L., Gill, J.H., 1996. Management of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. Int. J. Parasitol. 26, 993–1000.
Donald, A.O., Waller, P.J., Dobson, R.J., Axelson, A., 1980. The effect of selection with levamisole on benzimidazole resistance in *Ostertagia* spp. infections of sheep. Int. J. Parasitol. 10, 381–389.
Douvres, F.W., 1957. The morphogenesis of the parasitic stages of *Trichostrongylus axei* and *Trichostrongylus colubriformis* nematode parasites of cattle. Proc. Helminth. Soc. Wash. 24, 4–14.
Elmahalawy, S.T., Halvarsson, P., Skarin, M., Höglund, J., 2018. Droplet digital polymerase chain reaction (ddPCR) as a novel method for absolute quantification of major gastrointestinal nematodes in sheep. Vet. Parasitol. 15, 1–8.
Eysker, M., 1981. Experiments on inhibited development of *Haemonchus contortus* and *Ostertagia circumcincta* in sheep in The Netherlands. Res. Vet. Sci. (1), 62–65.
Eysker, M., 1997. The sensitivity of the Baermann method for the diagnosis of primary *Dictyocaulus viviparus* infections in calves. Vet. Parasitol. 69, 89–93.
Fernández, A.S., Fiel, C.A., Steffan, P.E., 1999. Study on the inductive factors of hypobiosis of *Ostertagia ostertagi* in cattle. Vet. Parasitol. 81, 295–307.
Fernando, S.T., 1965. Morphology, systematics and geographical distribution of *Mecistocirrus digitatus,* a trichostrongylid parasite of ruminants. J. Parasitol. 51, 149–155.

Forbes, A.B., Reddick, D., Stear, M.J., 2015. Efficacy of treatment of cattle for liver fluke at housing: influence of differences in flukicidal activity against juvenile *Fasciola hepatica*. Vet. Rec. 176, 333.
Francis, E.K., Slapeta, F.J., 2022. A new diagnostic approach to fast track and increase the accessibility of gastrointestinal nematode identification from faeces: FECPAK^{G2} egg nemabiome metabarcoding. Int. J. Parasitol. 52, 331–342.
Gasser, R.B., Chilton, N.B., Hoste, H., Beveridge, I., 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Res 21, 2525–2526.
Gasser, R.B., Chilton, N.B., Hoste, H., Stevenson, L.A., 1994. Species identification of trichostrongyle nematodes by pcr-linked rflp. Int. J. Parasitol. 24, 291-193.
Geary, T.G., Hosking, B.C., Skuce, P.J., von Samson-Himmelstjerna, G., Maeder, S., Holdsworth, P., Pomroy, W., Vercruysse, J., 2012. World Association for the Advancement of Veterinary Parasitology (WAAVP) Guideline: anthelmintic combination products targeting nematode infections of ruminants and horses. Vet. Parasitol. 190, 306–316.
Geurden, T., Smith, E.R., Vercruysse, J., Yazwinski, T., Settje, T., Nielsen, M.K., 2022. World association for the advancement of veterinary parasitology (WAAVP) guideline for the evaluation of the efficacy of anthelmintics in food-producing and companion animals: general guidelines. Vet. Parasitol. 304, 109698.
Halvarsson, P., Höglund, J., 2021. Sheep nemabiome diversity and its response to anthelmintic treatment in Swedish sheep herds. Parasit. Vectors 14, 114.
Herd, R.P., 1971. The parasitic life cycle of *Chabertia ovina,* in sheep. Int. J. Parasitol. 1 189–199.
Herlich, H., 1954. The life history of *Nematodirus helvetianus*, a nematode parasite in cattle. J. Parasitol. 40, 60–70.
Högberg, N., Baltrusis, P., Enweji, N., Höglund, J., 2022. Assessment of three DNA extraction kits for the absolute quantification of strongyle nematode eggs in faecal samples. Acta Vet. Scand. 64, 5.
Höglund, J., Elmahalawy, S.T., Halvarsson, P., Gustafsson, K., 2019. Detection of *Haemonchus contortus* on sheep farms increases using an enhanced sampling protocol combined with PCR based diagnostics. Vet. Parasitol. 276, 100018.
Höglund, J., Engström, A., von Samson-Himmelstjerna, G., Demeler, J., Tydén, E., 2013. Real-time PCR detection for quantification of infection levels with *Ostertagia ostertagi* and *Cooperia oncophora* in cattle faeces. Vet. Parasitol. 197, 251–257.
Horak, I.G., 1967. Host-parasite relationships of *Paramphistomum microbothrium* Fischoeder, 1901, in experimentally infested ruminants, with particular reference to sheep. Onderstepoort J. Vet. Res. 34, 451–540.
Horak, I.G., 1971. Immunity in *Gaigeria pachyscelis* infestation. J. S. Afr. Vet. Med. Assoc. 42, 149–153.
Kaplan, R.M., Denwood, M.J., Nielsen, M.K., Thamsborg, S.M., Torgerson, P.R., Gilleard, J.S., Dobson, R.J., Vercruysse, J., Levecke, B., 2022. World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for diagnosing anthelmintic resistance using the faecal egg count reduction test in ruminants, horses and swine. Vet. Parasitol. 318, 109936.
Kates, K.C., Turner, J.H., 1955. Observations on the life cycle of *Nematodirus spathiger*, a nematode parasite in the intestine of sheep and other ruminants. Am. J. Vet. Res. 16, 105–115.
Keith, R.K., 1967. The life history of *Cooperia pectinata*. Aust. J. Zool. 15, 739–744.
Laabs, E.M., Schnieder, T., Strube, C., 2012. Transcriptional differences between hypobiotic and non-hypobiotic preadult larvae of the bovine lungworm *Dictyocaulus viviparus*. Parasitol. Res. 110, 151–159.
Leathwick, D.M., Besier, R.B., 2014. The management of anthelmintic resistance in grazing ruminants in Australasia – strategies and experiences. Vet. Parasitol. 204, 44–54.
Eysker, M., Boersema, J.H., Hendrikx, W.M.L., 1990. Recovery of different stages of *Dictyocaluus viviparus* from cattle by a combination of a perfusion and a Baermann technique. Res. Vet. Sci. 49, 374–375.
McKenna, P.B., 1981. The diagnostic value and interpretation of faecal egg counts in sheep. N. Z. Vet. J. 29, 129–132.
Ministry of Agriculture, Fisheries and Food (MAFF), 1986. Manual of Veterinary Parasitological Laboratory Techniques. Reference Book N. 418, 3rd. ed. Her Majesty's Stationary Office, London.
Mitchell, G., Zadoks, R.N., Skuce, P.J., 2021. A universal approach to molecular identification of rumen fluke species across hosts, continents, and sample types. Front. Vet. Sci. 7, 605259.
Mönnig, H.O., 1927. The life history of *Trichostrongylus instabilis* and *T. rugatus* of sheep in South Africa. Rep. Vet. Res. Un. S. Afr 11/12, 229–251.
Murrell, K.D., Leighton, E.A., Boswell, B.A., Gasbarre, L.C., 1989. Comparison of egg excretion and serum pepsinogen levels as measures of nematode worm burdens in calves with limited pasture exposure. J. Parasitol. 75, 360–366.
Nielsen, M.K., 2021. What makes a good fecal egg count technique? Vet. Parasitol. 296, 109509.
Oakley, G.A., 1980. The recovery of *Dictyocaulus viviparus* from bovine lungs by perfusion: a modification of lnderbitzin's method. Res. Vet. Sci. 29, 395–396.
Ortlepp, R.J., 1937. Observations on the morphology and life history of *Gaigeria pachyscelis;* a hookworm parasite of sheep and goats. Onderstepoort J. Vet. Sci. Anim. Ind. 8, 183–212.
Queiroz, C., Levy, M., Avramenko, R., Redman, E., Kearns, K., Swain, L., Silas, H., Uehlinger, F., Gilleard, J.S., 2020. The use of ITS-2 rDNA nemabiome metabarcoding to enhance anthelmintic resistance diagnosis and surveillance of ovine gastrointestinal nematodes. Int. J. Parasitol. Drugs Drug. Resist. 14, 105–117.
Redman, E., Queiroz, C., Bartley, D.J., Levy, M., Avramenko, R.W., Gilleard, J.S., 2019. Validation of ITS-2 rDNA nemabiome sequencing for ovine gastrointestinal nematodes and its application to a large scale survey of UK sheep farms. Vet. Parasitol. 275, 108933.
Rehbein, S., Visser, M., 2002. Efficacy of Ivermectin delivered via a controlled-release capsule against small lungworms (Protostrongylidae) in sheep. J. Vet. Med. 49, 313–316.
Reinecke R.K. 1973. The larval anthelmintic test in ruminants. Technical Communication No. 106, Dept. of Ag. Technical Services, Rep. of South Africa.
Reinecke, R.K., 1980. Chemotherapy in the control of helminthosis. Vet. Parasitol. 6, 255–292.
Rinaldi, L., Veneziano, V., Morgoglioni, M.E., Pennacchio, M., Santaniello, M., Schioppi, V., Musella, V., Fedele, V., Cringoli, G., 2009. Is gastrointestinal strongyle faecal egg count influenced by hour of sample collection and worm burden in goats? Vet. Parasitol. 163, 81–86.
Rode, B., Jorgensen, R.J., 1989. Baermannization of *Dictyocaulus* spp. from faeces of cattle, sheep and donkeys. Vet. Parasitol. 30, 205–211.
Roeber, F., Hassan, E.B., Skuce, P., Morrison, A., Claerebout, E., Casaert, S., Homer, D.R., Firestone, S., Stevenson, M., Smith, L., Larsen, J., 2017. An automated, multiplex-tandem PCR platform for the diagnosis of gastrointestinal nematode infections in cattle: an Australian-European validation study. Vet. Parasitol. 239, 62–75.
Roeber, F., Jex, A.R., Gasser, R.B., 2013. Comparative evaluation of two DNA isolation techniques for PCR-based diagnosis of gastrointestinal nematode infections in sheep. Mol. Cell. Probes 27, 153–157.
Roeber, F., Morrison, A., Casaert, S., Smith, L., Claerebout, E., Skuce, P., 2017. Multiplexed-tandem PCR for the specific diagnosis of gastrointestinal nematode infections in sheep: a European validation study. Parasit. Vectors 10, 226.
Rojas, C.A.V., Jex, A.R., Gasser, R.B., Scheerlinck, J.-P.Y., 2014. Techniques for the diagnosis of *Fasciola* infections in animals: room for improvement. Adv. Parasitol. 85, 65–107.
Rolfe, P.F., Boray, J.C., 1987. Chemotherapy of paramphistomosis in cattle. Aust. Vet. J. 64, 328–332.
Rose, J.R., 1969. The development of parasitic stages of *Ostertagia ostertagi*. J. Helminthol. 43, 173–184.
Santos, L.L., Salgado, J.A., Drummond, M.G., Bastianetto, E., Santos, C.P., Brasil, C.A., Taconeli, C.A., Oliveira, D.A.A., 2020. Molecular method for the semiquantitative identification of gastrointestinal nematodes in domestic ruminants. Parasitol. Res. 119, 529–543.
Sprent, J.F.A., 1946. Studies on the life history of *Bunostomum phlebotomum,* a hookworm parasite of cattle. Parasitology 37, 192–201.
Sweeny, J.P., Robertson, I.D., Ryan, U.M., Jacobson, C., Woodgate, R.G., 2011. Comparison of molecular and McMaster microscopy techniques to confirm the presence of naturally acquired strongylid nematode infections in sheep. Mol. Biochem. Parasitol. 180, 62–67.
Thomas, D.R., Probert, A.J., 1993. A key to the identification of arrested gastrointestinal nematode larvae of sheep in Britain. Vet. Parasitol. 47, 77–80.
Torgerson, P.R., Schnyder, M., Hertzberg, H., 2005. Detection of anthelmintic resistance: a comparison of mathematical techniques. Vet. Parasitol. 128, 291–298.
Turner, J.H., Shalkop, W.T., Wilson, G.I., 1960. Experimental strongylidiasis in sheep and goats. IV Migration of *Strongyloides papillosus* in lambs and accompanying pathological changes following percutaneous infection. Am. J. Vet. Res. 21, 536–546.
Ueno H., Gonçalves P., 1998. Manual para diagnóstico das helmintoses de ruminantes. 4ª Edición. Brasil: Japan International Cooperation Agency.
Van Wyk, J.A., Cabaret, J., Michael, L.M., 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. Vet. Parasitol. 119, 277–306.
Van Wyk, J.A., Gerber, H.M., Alves, R.M.R., 1984. Methods of infecting sheep with gastro-intestinal nematodes after cryopreservation: dosing of larvae in gelatin capsules compared to dosing of larvae in water suspension. Onderstepoort J. Vet. Res. 51, 217–221.
Van Wyk, J.A., Mayhew, E., 2013. Morphological identification of parasitic nematode larvae of small ruminants and cattle: a practical lab guide. Onderstepoort J. Vet. Res. 80, 539–553.
Veglia, F., 1915. The anatomy and life-history of the *Haemonchus contortus.* 3rd and 4th Rep. Dir. Vet. Ed. Res. Un. S. Afr. 347–500.
Veglia, F., 1923. Preliminary notes on the life history of Oesophagostomum columbianum. 9th and 10th Rep. Dir. Vet. Ed. Res. Un. S. Afr., 809-823.9th and 10th Reports of the Director of Vet. Educ. and Res., Dep.Agric., South Africa (1923), pp. 811-823.
Verster, A., Collins, H.M., Anderson, P.J.S., 1971. Studies on *Dictyocaulus filaria.* 1V. The morphogenesis of the parasitic stages in lambs. Onderstepoort J. Vet. Res 38, 199–206.
Wilkes, C.P., Thompson, F.J., Gardiner, M.P., Paterson, S., Viney, M.E., 2004. The effect of the host immune response on the parasitic nematode Strongyloides ratti. Parasitology 128, 661–669.
Williams, J.C., Knox, J.W., Baumann, B.A., Snider, T.G., Kimball, M.D., Hoerner, T.J., 1981. Anthelmintic activity of albendazole against inhibited larvae of *Ostertagia ostertagi*. Am. J. Vet. Res. 42, 2077–2080.
Williams, J.C., Knox, J.W., Sheehan, D.S., Fuselier, R.H., 1979. Activity of fenbendazole against inhibited early fourth-stage larvae *Ostertagia ostertagi*. Am. J. Vet. Res. 40, 1087–1090.
Wimmer, B., Craig, B.H., Pilkington, J.G., Pemberton, J.M., 2004. Non-invasive assessment of parasitic nematode species diversity in wild Soay sheep using molecular markers. Int. J. Parasitol. 34, 625–631.
Wolff, K., Ruosch, W., Eckert, J., 1969. A perfusion technique for the recovery of *Dicrocoelium dendriticum* from livers of sheep and cattle. Z. Parasite 33, 85–88.
Wood, L.B., Amaral, N.K., Bairden, K., Duncan, C., Kassai, T., Malone, J.B., Pankavich, J. A., Reinecke, R.K., Slocombe, O., Taylor, S.M., Vercruysse, J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of

guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.
Yazwinski, T.A., 1988. Use of febantel or ivermectin for treatment of calves with experimentally induced *Bunostomum phlebotomum* infection. Am. J. Vet. Res. 49, 1407–1408.

D.J. Burden[a,*], D.J. Bartley[b], R.B. Besier[c], E. Claerebout[d], T.P. Elliott[e], J. Höglund[f], S. Rehbein[g], J.F.J. Torres-Acosta[h], J.A. Van Wyk[i], T. Yazwinski[j]

[a] *Duilio Veterinary Parasitology, The Vicarage, Church Lane, Churcham, Gloucester, UK*
[b] *Disease Control, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, UK*
[c] *College of Environmental and Life Sciences, Murdoch University, Perth, WA, Australia*
[d] *Laboratory for Parasitology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium*
[e] *Centre for Animal Research and Teaching, University of New England, Armidale, NSW, Australia*
[f] *Section for Parasitology, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden*
[g] *Boehringer Ingelheim Vetmedica GmbH, Kathrinenhof Research Center, Rohrdorf, Germany*
[h] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico*
[i] *Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa*
[j] *University of Arkansas, Fayetteville, AR, USA*

[*] Corresponding author.
E-mail address: davejburden@msn.com (D.J. Burden).