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World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guideline for diagnosing anthelmintic resistance using the faecal egg count reduction test in ruminants, horses and swine

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ABSTRACT

The faecal egg count reduction test (FECRT) remains the method of choice for establishing the efficacy of anthelmintic compounds in the field, including the diagnosis of anthelmintic resistance. We present a guideline for improving the standardization and performance of the FECRT that has four sections. In the first section, we address the major issues relevant to experimental design, choice of faecal egg count (FEC) method, statistical analysis, and interpretation of the FECRT results. In the second section, we make a series of general recommendations that are applicable across all animals addressed in this guideline. In the third section, we provide separate guidance details for cattle, small ruminants (sheep and goats), horses and pigs to address the issues that are specific to the different animal types. Finally, we provide overviews of the specific details required to conduct an FECRT for each of the different host species. To address the issues of statistical power vs. practicality, we also provide two separate options for each animal species; (i) a version designed to detect small changes in efficacy that is intended for use in scientific studies, and (ii) a less resource-intensive version intended for routine use by veterinarians and livestock owners to detect larger changes in efficacy. Compared to the previous FECRT recommendations, four important differences are noted. First, it is now generally recommended to perform the FECRT based on pre- and post-treatment FEC of the same animals (paired study design), rather than on post-treatment FEC of both treated and untreated (control) animals (unpaired study design). Second, instead of requiring a minimum mean FEC (expressed in eggs per gram (EPG)) of the group to be tested, the new requirement is for a minimum total number of eggs to be counted under the microscope (cumulative number of eggs counted before the application of a conversion factor). Third, we provide flexibility in the required size of the treatment group by presenting three separate options that depend on the (expected) number of eggs counted. Finally, these guidelines address all major livestock species, and the thresholds for defining reduced efficacy are adapted and aligned to host species, anthelmintic drug and parasite species. In conclusion, these new guidelines provide improved methodology and standardization of the FECRT for all major livestock species.

1. Introduction

The periodic administration of anthelmintic drugs is currently the most widely used method to control gastrointestinal nematode infections in livestock animals. This control strategy focuses on reducing infection intensity and transmission to prevent production losses and to minimize the risk of parasitic disease (Shaw et al., 1998; Charlier et al., 2009, 2020). The three major classes of anthelmintics are the

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benzimidazoles (BZ), imidazothiazoles/tetrahydropyrimidines (IT), and the macrocyclic lactones (ML), which make up the majority of anthelmintic products used. Additionally, there are two newer drug classes: the amino-acetonitrile derivatives (AADs, e.g. monepantel) and spiroindoles (e.g. derquantel). However, in most countries these new drug classes are only registered for use in sheep. The intensive use of anthelmintics for controlling helminth parasites has led to high levels of anthelmintic resistance (AR) worldwide in almost all major parasite species infecting all types of livestock (Kaplan, 2004; Sutherland and Leathwick, 2011; Matthews, 2014; Ramünke et al., 2016; Traversa and von Samson-Himmelstjerna, 2016). Consequently, monitoring the efficacy of anthelmintic drugs is an important component of animal health and production management (Kaplan and Vidyashankar, 2012).

Anthelmintic resistance is a heritable trait (Prichard et al., 1980), and is defined as occurring 'when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of an anthelmintic drug, are no longer affected, or a greater concentration of drug is required to reach a certain level of efficacy' (Wolstenholme et al., 2004). However, many factors other than AR can be the cause of reduced efficacy, and can therefore impact the results of a faecal egg count reduction test (FECRT) (Supplementary file 1) (Vidyashankar et al., 2007; Vidyashankar et al., 2012; Morgan et al., 2022). Consequently, efficacy data can only be correctly interpreted, and a diagnosis of AR be correctly made, if a number of other factors can be eliminated as a source of the reduced efficacy. See Box 1 for a list of conditions that must be met before one can conclude that an observation of reduced efficacy is consistent with a diagnosis of AR.

A large number of tests have been described to assess AR, including in vivo (FECRT and controlled efficacy test [measurement of worm counts at necropsy]), in vitro (larval migration inhibition test, motility-based tests, larval development test, larval feeding test and egg hatch test) and molecular tests (Taylor et al., 2002; Coles et al., 2006; Avramenko et al., 2019). Though several in vitro tests have proven valuable in some research settings, these tests have limited application and usefulness for diagnosis of AR at the farm level. Some of the logistical and practical problems associated with these tests include: lack of validation in many hosts and parasite species, narrow spectrum with regard to drug class, the requirement for technical expertise and or specialized equipment, and difficulty in standardization between laboratories. Consequently, the FECRT remains the method of choice to assess drug efficacy, and hence the most commonly applied test for the diagnosis of AR (Kaplan and Vidyashankar, 2012). In contrast to the other tests, the FECRT permits the assessment of drug efficacy for all anthelmintic classes across all animal species, and for multiple parasite species without sacrificing the animals. Furthermore, it can be done locally without the need for a reference diagnostic laboratory or specialized equipment and/or expertise.

Previously, methods and recommendations for conducting an FECRT were provided in the World Association for the Advancement of Veterinary Parasitology (WAAVP) publication on detecting AR in nematodes of veterinary importance (Coles et al., 1992). These recommendations were primarily designed for use in sheep with few details or specific recommendations being provided for horses, cattle and pigs. However, there are important host-specific and parasite-specific differences that require protocol modifications to address these distinctions. Additionally, since the publication of those recommendations in the early 1990 s, many new insights have been gained regarding the optimal experimental design for the FECRT (Torgerson et al., 2005; Levecke et al., 2012; Levecke et al., 2018), the impact of variability on the ability to make accurate interpretations of FECRT data (Levecke et al., 2012; Vidyashankar et al., 2012) and the optimal methods for statistical analysis of FECRT data (Dobson et al., 2009; Dobson et al., 2012; Denwood et al., 2019). This improved understanding has yielded new recommendations and statistical tools for improving the design and analysis of FECRT studies (Wang et al., 2018; Denwood et al., 2019).

Recent work also illustrates that accurately determining whether a reduction in efficacy is sufficient to make a classification of resistance is quite difficult when the observed efficacy is close to the classification threshold (Levecke et al., 2012; Levecke et al., 2018; Denwood et al., 2023). Consequently, it is not possible to design an FECRT that is both simple and practical to perform, and is also computationally robust in its ability to provide correct classifications of resistance status. Furthermore, there is a strong demand among the veterinary parasitology community for a simplified guideline that will facilitate the performance of FECRT as a farm-level diagnostic.

In consideration of these multiple issues, we have developed a guideline that has four sections. In the first section, we address general considerations relevant to the major issues germane to the performance of the FECRT, such as experimental design, choice of faecal egg count (FEC) method, and statistical analysis of FECRT data along with interpretation of the FECRT results. In the second section, we provide an operational overview for carrying out the FECRT that is relevant to all host animals. In the third section, we provide host-specific guidance that addresses many of the issues that are relatively unique to each major

Box 1
Conditions that must all be true before one can conclude that reduced efficacy in an FECRT is consistent with a diagnosis of anthelmintic resistance.

1. Animals were treated with the proper dose (all animals received the minimum label dosage).
2. Animals were treated using a proper administration technique.
3. Anthelmintic drug was used within the expiration date and was properly stored prior to using in the FECRT.
4. The same animals were sampled both pre- and post-treatment with an interval appropriate for the anthelmintic used, and animals were correctly identified.
5. Both pre-treatment and post-treatment faecal samples were freshly collected, and labelled and stored correctly.
6. A faecal egg count method suitable for the FECRT was applied and proper laboratory techniques were used.
7. The drug being tested had been previously demonstrated to be efficacious against the target parasite species in the same host animals when administered at the dosage being tested.
8. Adequate numbers of animals were tested and adequate numbers of eggs were counted in the pre-treatment samples.
9. An appropriate statistical method was used, and the statistical results indicated sufficient confidence in the repeatability of the result (i.e. confidence intervals were calculated using an appropriate method and these were used for making the diagnosis).
10. The quality of the anthelmintic product can be assured.

animal group. Finally, in the fourth section, we provide simple host and parasite species-specific guidance regarding group sizes and the number of counted eggs required for performing a standardized FECRT on cattle, small ruminants (sheep and goats), horses and pigs. In addition, to address the issues of complexity vs. practicality, we provide two separate options for each animal species, (i) a version designed to detect small changes in efficacy that is intended for use in scientific studies ('research protocol'), and (ii) a version with less stringent experimental requirements ('clinical protocol') that is intended for clinical use by veterinarians and livestock owners to detect larger changes in efficacy. More details on the difference between the two protocols are provided in Section 1. However, it is important to note here that both approaches/protocols are designed to limit false positive determinations of resistance or susceptibility to the same type 1 error rate, and so can be considered equally robust from that perspective. However, the 'clinical protocol' will more often yield inconclusive results as compared to the 'research protocol' as a result of the lower statistical power to detect small changes in efficacy (Denwood et al., 2023).

Compared to the previous recommendations (Coles et al., 1992), four important differences are noted. First, it is now recommended to perform the FECRT based on pre- and post-treatment FEC of the same animals, rather than on post-treatment FEC of both treated and untreated (control) animals. Second, instead of requiring a minimum mean FEC in the group to be tested, the new requirement is for a minimum total number of eggs to be counted under the microscope; i.e. the sum of the raw counts of eggs before application of a conversion factor. This change was required because the mean EPG has little bearing on the measurement of efficacy; it is the total number of eggs counted in the pre-treatment and post-treatment FEC that has quantitative relevance. Requiring that a minimum number of eggs be counted in the pre-treatment FEC not only improves the ability to accurately detect a true reduction in efficacy, it also avoids the need to dictate which FEC method should be used. Rather, one of several different FEC methods can be selected depending on relevant factors such as the mean FEC of the group and the number of animals being tested. Third, we provide three options for the required size of the treatment group depending on the number of eggs counted (which considers both the starting mean FEC of the animal group being tested and the multiplication factor of the FEC method used). Thus, if the approximate starting mean FEC of the animal group being tested is known in advance, and is relatively high, then fewer animals need to be tested or a FEC method with a higher multiplication factor can be used. This approach provides greater flexibility and precision for making accurate classifications of drug efficacy compared to the previous recommendations. Finally, additional target thresholds for classification of drug efficacy are provided for horses and pigs with regard to both drug and parasite species, as the original published efficacy data differs from that of ruminants.

In conclusion, these new guidelines provide improved methodology and standardization of the FECRT for all major livestock species. Additionally, by providing two options for each animal species, we accept that there are important statistical challenges presented by FECRT data, but do not let these issues prevent us from developing a guideline that is flexible, and also practical for livestock owners and veterinarians. These new guidelines will therefore provide researchers, the pharmaceutical industry, government agencies, veterinarians and livestock owners with standardized approaches that will improve both the measurement of FECR and the classification of AR status.

2. Section 2: factors and insights relevant to the performance of the FECRT

It is important to distinguish the measurement of FEC reduction from an FECRT. FEC reduction is simply a calculation of the percent reduction in egg counts following anthelmintic treatment, without qualifiers for how the measurement was done, or how accurate it might be. In contrast, an FECRT is a field-based diagnostic test designed to not only measure the level of reduction, but to also permit the introduction of diagnostic criteria that have statistical validity and clinical relevance. There are a number of factors that must be considered when planning an FECRT; these can be broadly subdivided into issues related to (i) study design, (ii) sample size considerations, (iii) choice of FEC methods, (iv) statistical data analysis, and (v) interpretation. These are summarized separately in this section.

2.1. Study designs for FECRT

There are two main designs for the FECRT: those employing separate treatment and control animals (unpaired study design) and those using pre- and post-treatment samples from the same animals (paired study design). The previous WAAVP methods for performing FECRTs (Coles et al., 1992) recommended an unpaired design using a non-treated control group. However, unless pre-assignment FEC are performed on all animals in order to balance the mean EPG of the groups, then differences in FECs across treatment groups at baseline may considerably compromise the FECR estimates based on control animals solely due to randomness (Torgerson et al., 2005). Moreover, several sheep studies found that including control animals in FECRT did not improve the sensitivity of diagnosing AR (McKenna, 2006; Dobson et al., 2012; Calvete and Uriarte, 2013), and in a retrospective study, McKenna (2006) found that analysis of FECRT data using pre- and post-treatment counts from the same animals (paired study design) was more sensitive (95.9 %) in detecting AR than when control animals (unpaired study design) were included (91.2 %). Simulation studies that were performed in parallel with the current document also confirmed that a paired study design using pre- and post-treatment counts from the same animals yields conclusive results more often than unpaired studies (Denwood et al., 2023). In addition to these evidence-based arguments, there are practical issues that hamper the inclusion of a control group. In some cases, there are insufficient numbers of animals to allocate to different treatment groups or there is the reluctance of the livestock owners to leave animals untreated. Consequently, to maximize the standardization, accuracy and precision of the FECRT, it is now recommended to base an FECRT on pre- and post-treatment FECs of the same animals. However, this approach inherently assumes that the sole temporal trend in mean count between successive FEC is due to the effect of treatment. This approach is therefore not advised for situations where age-related or strong seasonal trends in mean FEC are expected, such as for *Parascaris* spp. in foals.

Another design consideration is the use of composite faecal samples rather than individual animal samples. The use of composite samples can reduce the laboratory effort and cost associated with an FECRT, and will frequently provide the same diagnosis as individual samples (Rinaldi et al., 2014; Kenyon et al., 2016; George et al., 2017). However, none of the standard methods that are currently available for analysing FECRT have been validated statistically for use with composite samples. Some work has been done to examine the number of animals that should be included when using composite counts for basing anthelmintic dosing decisions in sheep (Morgan et al., 2005), and further work has provided evidence for a strong correlation between individual-based and composite-based FECRT (George et al., 2017). However, there are a number of additional factors that must be taken into consideration for assessing the statistical confidence of an FECRT result, including: how to deal with missing post-treatment samples and how to calculate confidence intervals (CI) for the FECR from the resulting data. Composite counts may also conceal the effect of mis-dosing of a single individual, as it is impossible to determine if a moderately high post-treatment composite FEC is driven by a single animal with extremely high FEC. Morgan et al. (2005) also noted that higher levels of over-dispersion (k) may complicate the interpretation of composite samples by masking the contribution of higher-shedding animals. We note that consideration of these issues warrants further study, and therefore we do not provide a specific protocol for composite samples in these guidelines.

2.2. Sample size considerations

An important consideration of planning an FECRT is to ensure that the proposed sample size is sufficient for a conclusive result to be obtainable. A convenient mechanism for doing this is the adaption of the familiar concept of statistical power to the situation for FECRT, whereby it is ensured that we simultaneously have ≥80 % power for the following outcomes from the two related but distinct statistical tests that are relevant to a FECRT:

(i) positive evidence of 'resistance' should the efficacy be reduced, and
(ii) positive evidence of 'susceptibility' should the efficacy be adequate.

A core aim of these guidelines is to stipulate a sample size that is sufficient to obtain ≥80 % power for both statistical tests, although this must necessarily depend on the host and parasite species under consideration, as well as the anthelmintic and protocol being used. A complete discussion of the factors relevant to statistical power of an FECRT are provided in (Denwood et al. (2023); here we provide a non-technical summary of the concepts necessary to understand the recommendations provided in these guidelines.

There are five broad issues that affect the overall statistical power of an FECRT:

(i) the number of animals (i.e., the sample size)
(ii) the total number of eggs counted
(iii) the variability in egg counts across animals prior to and after drug administration, as well as the within-animal correlation in egg counts between pre- and post-treatment datasets
(iv) the expected efficacy of the administered drug
(v) the lower efficacy threshold representing the smallest possible reduction in efficacy that can be reliably diagnosed as 'resistant', where the interval between lower efficacy threshold and expected efficacy may be referred to as the 'grey zone'.

Of these, the sample size may be increased or decreased as necessary to maintain sufficient power, within the practical limitations of the available number of animals. However, we strongly suggest that a minimum sample size requirement of five animals always be maintained. This is necessary because an estimate of variability is required for currently available data analysis methods, and this estimate will become unreliable with fewer than five observations. The total number of eggs counted is affected by a combination of the mean FEC and the method used to enumerate eggs, and is therefore also partly under the control of the investigator. Conversely, the biological variability in mean egg counts (i.e., excluding the variability arising from the Poisson distribution due to the counting process) and within-animal correlation are fixed by the biology of the system, and must therefore be estimated based on previous experience of the same hosts and parasites for use within power calculations. Similarly, the expected efficacy of the administered drug should correspond to published efficacy estimates in relatively drug-naïve populations of nematode parasites, and can therefore also be assumed to be fixed.

The final factor to consider is the width of the 'grey zone' below the expected efficacy, which is determined by the lower efficacy threshold, and derives the range of observed efficacies within which we can expect an inconclusive result from the FECRT. This grey zone represents a deliberate trade-off between logistical considerations and the expected sensitivity to detect small reductions in efficacy and is the basis of the difference between the 'clinical protocol' and 'research protocol' given in these guidelines. For example, for sheep we have an expected efficacy of 99 % and we may choose a research protocol with a grey zone of 95–99 % or a clinical protocol with a grey zone of 90–99 % (see Denwood et al., 2023 for an illustrative example). With the former, we can expect positive evidence of resistance with a reduction of 95 %, but at the cost of a high sample size requirement. Conversely, with the latter the minimum detectable resistance drops to 90 %, but the sample size requirements are reduced. Explicit consideration of the so-called 'non-inferiority margin', that determines this lower efficacy threshold is an important improvement over the previous guidelines and allows for simultaneous provision of research and clinical protocols that are focussed on different resource availability and clinical objectives. For more details on the use of non-inferiority testing within this context see Denwood et al. (2023), and for discussion of the choice of non-inferiority margin within a medical context see Walker and Nowacki (2011).

2.3. Choice of FEC method

There are a variety of laboratory methods that are commonly used to perform FECs. These include the traditional microscopy-based methods such as the McMaster method (Ministry of Agriculture, 1986), concentration McMaster method (Roepstorff and Nansen, 1998), Cornell-Wisconsin method (Egwang and Slocombe, 1982), the modified Stoll method (Stoll, 1930), and the FLOTAC (Cringoli et al., 2010) and Mini-FLOTAC methods (Cringoli et al., 2017). Additionally, there are several new semi-automated, e.g. FECPAKG2 (Rashid et al., 2018; Ayana et al., 2019) and fully automated image analysis-based methods, e.g. Parasight, VETSCAN IMAGYST and Telenostic systems (Cain et al., 2020; Elghryani et al., 2020; Nagamori et al., 2020) and other systems are likely to be developed in the future. These various diagnostic methods differ considerably in their multiplication factor, accuracy (degree of deviation from the true FEC), precision (degree of repeatability of FECs performed on the same faecal sample), and in terms of their need for financial, human and technical resources under field and laboratory conditions (Levecke et al., 2009; Van den Putte et al., 2016; Paras et al., 2018).

Although these factors will impact the decision of which FEC method to use, it is well established that the precision of FECRT results improves when the number of animals tested increases and the multiplication factor of the FEC method decreases (i.e. the number of eggs counted under the microscope increases) (Levecke et al., 2011; Torgerson et al., 2012; Calvete and Uriarte, 2013). Consequently, for the purpose of performing an FECRT, the optimal choice of FEC method will largely depend on the number of animals and the mean FEC (in EPG) of the test group (Levecke et al., 2012). However, for the following reasons, it is not advisable to uniformly recommend one specific FEC method/protocol: (i) the increased financial and technical support required for some of the FEC methods with low multiplication factors, (ii) the large variation in egg excretion both between host and parasite species, (iii) the difference in number of animals available for testing in an FECRT across host species (e.g. sheep vs. horses), and (iv) the large variation in protocols of the same FEC method across different laboratories (Cringoli et al., 2004; Pereckiene et al., 2007; Vadlejch et al., 2011). While these factors should be considered carefully before choosing a method for a particular FECRT study, methods with lower multiplication factors are generally recommended. However, it should be emphasized that methods based on centrifugation of test tubes and subsequent reading of cover slips, such as the Wisconsin method, generally perform with substantially lower accuracy and precision levels than counting chamber based methods like McMaster and Mini-FLOTAC (Bosco et al., 2018; Paras et al., 2018; Cain et al., 2020), and are therefore generally not recommended for FECRT studies.

2.4. Thresholds for evaluating FECR data

A key factor in making a diagnosis of AR is the expected efficacy in fully susceptible populations, as per when the drugs were first introduced. A diagnosis of AR can only be made under two conditions: firstly that one can demonstrate with relative statistical certainty (accepting a 5 % type 1 error rate) that the true efficacy in the sampled population is

really less than the expected efficacy for the same anthelmintic compound applied to a population of susceptible parasites, and secondly that other factors not associated with AR, but that could potentially cause a reduction in efficacy, are eliminated as potential causes (see Box 1). Thus, developing evidence-based guidelines for FECRT requires having strong evidence of the original baseline efficacy for each drug and host, and in some cases for each parasite, to be able to make appropriate conclusions regarding true anthelmintic resistance.

This explicit consideration of the expected efficacy of an anthelmintic compound under the situation of 'susceptibility' is a new addition to these guidelines. In the previous WAAVP recommendations for FECRT (Coles et al., 1992), AR was considered to be present in sheep (for all drugs) if the mean FECR was less than 95 % and the lower 95 % CI for FECR was less than 90 %. For horses, cattle and swine, it was suggested that a mean FECR of less than 90 % was indicative of AR, with no recommendations for using CI in drawing an inference. Additionally, for horses this recommendation was confined to BZ, with no recommendations for the other drugs.

In preparing these new guidelines, our data analyses revealed that the protocol requirements for an FECRT were vastly different depending on whether the expected efficacy was 99.9, 99, 98, 95 or 90 %. Thus, we searched the literature to establish what the reported levels of FECR were for each host, parasite and anthelmintic at the time of the registration studies, and other efficacy studies performed early in the commercial life of the drugs. We determined that for ruminants, virtually all studies for all anthelmintics demonstrated greater than 99 % reduction in worm counts and/or FEC (Herlich, 1977; Malan, 1981; Benz et al., 1989; Entrocasso et al., 1996; Meeus et al., 1997), and thus, we selected 99 % as the target efficacy for these guidelines for sheep, goats, and cattle for all of the anthelmintics addressed in this document. For horses and swine, there were large and distinct differences in the levels of FECR among the various anthelmintics and major parasite species. Consequently, in these guidelines, for horses and pigs we recommend the use of different thresholds for different drugs and for different parasites. These data are provided in the host-specific section (Section 4) and the specific parameters for the FECRT section (Section 5).

2.5. Statistical data analysis

In order to interpret the result of an FECRT, it is necessary to obtain estimates of uncertainty in the observed reduction using a valid statistical method. Estimates of uncertainty are most likely to be based on a single statistical method yielding a lower and upper CI, but may alternatively be based on separate statistical tests to obtain *p*-values for paired non-inferiority and inferiority tests using a pre-specified expected efficacy and lower efficacy threshold or non-inferiority margin (Denwood et al., 2019). Either way, the statistical method used should have a 5 % type 1 error rate for classifications of both AR and susceptibility, as corresponding to the assumptions used for the power calculation. Importantly, this requires the use of a 90 % CI for all statistical methods producing CIs, as opposed to the 95 % CIs that have previously been used. The rationale for this is detailed by Denwood et al. (2023) but can be summarized as resulting from the use of two separate one-tailed tests for resistance and susceptibility. Unlike a two-tailed test, which corresponds to 2.5 % probability in each of the two tails as given by a 95 % CI, each one-tailed test requires a 5 % probability in a single tail of the distribution. This necessitates the use of a 90 % CI, with only the upper 90 % CI relevant to the test for resistance and only the lower 90 % CI relevant to the test for susceptibility. The important observation of Denwood et al. (2023) is that type I error outcomes for each of these tests are mutually exclusive, i.e. a reduction cannot be simultaneously 'false positive' for both resistance and susceptibility, so that the overall type I error rate is maintained at 5 % without requiring any correction for multiple testing. The practical benefit of this change is that fewer animals and counted eggs are needed to generate the required power to detect AR or susceptibility. It should also be noted that these two tests can also be used in isolation: for example the test for resistance may be used without the test for susceptibility for research studies where it is solely of interest to disprove the null hypothesis of adequate efficacy. However, this practice complicates the overall interpretation of the results of the FECRT and so is not addressed further here. For additional discussion on this issue see Denwood et al. (2023).

While the authors' recommendations are not restricted to any single method of analysis, it is important to note that the available methods for calculating CI are not equally appropriate for this purpose. A particular issue arises when the observed FECR is 100 %, as only a few of the available formulae can generate a CI when the observed efficacy is 100 % (Denwood et al., 2010; Dobson et al., 2012; Torgerson et al., 2014). A Bayesian framework provides additional analysis options, but erroneous inference can be obtained from the use of inappropriate assumptions, for example not accounting for variation in efficacy between animals (Levecke et al., 2018) or assuming that over-dispersion is unaffected by treatment (Pena-Espinoza et al., 2016). Due to the complicating issues involved in choosing an appropriate method, the authors suggest using a Bayesian approach that has been developed and tested for the specific purpose of analysing FECRT data (Denwood et al., 2010; Dobson et al., 2012; Torgerson et al., 2014; Wang et al., 2018; Denwood et al., 2019). This recommendation applies to all FECRT, but is most important when the observed efficacy is less than 100 %. Presently there are two web-based analysis tools that make analysing FECRT quite easy and straightforward, and it is recommended that one of these web sites be used unless a qualified statistician performs the data analysis. The eggCounts method (Torgerson et al., 2014; Wang et al., 2018) uses a Bayesian hierarchical model, http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/. A second approach (http://www.fecrt.com), provides a web application that uses a hybrid Frequentist/Bayesian analysis method and is also pre-configured with the parameter values discussed here to further facilitate use (Denwood et al., 2019; Denwood et al., 2023). Alternatively, when the observed efficacy is less than 100 %, and one chooses not to use one of the analysis applications mentioned above, there are several other methods that are appropriate for calculating CI (for an example see Supplementary file 2). It is also expected that further statistical methods and online tools will be developed within the lifetime of these guidelines, which may also provide further benefits compared to the currently available methods and tools.

The classification framework that the authors advocate is based exclusively on estimates of uncertainty in the true reduction (i.e. 90 % CI), rather than the 'observed' reduction (Denwood et al., 2023). Therefore, it is not necessary to stipulate a method for calculating the observed (mean) reduction in these guidelines. Given the complex discussions around the use of different calculation methods for the mean FECR, including choice of arithmetic vs. geometric means, we consider this new framework to be a substantial advantage.

2.6. Interpretation

The interpretation of an FECRT is rather straightforward using the classification criteria outlined by Denwood et al. (2023). These criteria are based on how the observed data (which yields values for the upper and lower 90 % CI or results of separate hypothesis tests) compare to the expected efficacy and to the lower efficacy threshold (which serve as the values for the upper and lower limits of the grey zone), accepting a 5 % type 1 error rate. Using these new criteria, we use the following approach to classify the results of an FECRT, which yields three possible classifications (see Denwood et al. (2023) for a graphical figure illustrating these classifications):

- *Susceptible:* when the lower 90 % CI is greater than or equal to the lower efficacy threshold (corresponding to the lower limit of the grey zone) and the upper 90 % CI is greater than or equal to the expected efficacy (corresponding to the upper limit of the grey zone).

- o *Simplified explanation:* the worst probable true efficacy is still within an acceptable margin of the expected efficacy, and the best probable true efficacy is equal to or greater than the expected efficacy.

- *Resistant:* when the upper 90 % CI is less than the expected efficacy (corresponding to the upper limit of the grey zone). This includes the sub-classification of 'low resistant', which meets the additional criteria of the lower 90 % CI being greater than or equal to the lower efficacy threshold (lower limit of the grey zone).
  - o *Simplified explanation:* the best probable true efficacy is less than the expected efficacy of the drug.
- *Inconclusive:* if neither of these criteria are met
  - o *Simplified explanation:* given the established grey zone, the data are not sufficient to make any positive conclusions regarding the true efficacy with respect to resistance or susceptibility.

Use of this classification scheme ensures that positive classifications of either susceptibility or resistance are based on positive evidence. This removes the scenario where a lack of evidence for susceptibility may be confused with a diagnosis of AR (Denwood et al., 2023). The provision of the *Inconclusive* classification provides a third outcome when the observed efficacy cannot be conclusively determined to be either significantly above the lower limit of the grey zone or significantly below the upper limit of the grey zone. Note that when the result of a test is inconclusive, the appropriate action to take will depend on the situation and goals of the test. If a conclusive outcome is desired, then it is advisable to repeat the FECRT using more animals and counting more eggs; doing so will increase the likelihood of obtaining a conclusive result.

Although presented as a sub-classification of '*resistant*' above, it is noted that '*low resistant*' could also be considered as an entirely separate fourth category where the criteria for both resistance and susceptibility are met simultaneously. In this case the true efficacy can be concluded to be somewhere within the grey zone, i.e. it is below the expected efficacy but also within an acceptable margin of that expected efficacy. We follow Denwood et al. (2023) in the desire to simplify the number of classifications to three and believe that '*resistant*' is the most biologically justifiable alternative classification for this rare event. It is noted however, that as a rare event this classification will rarely be seen, and thus, has very limited practical importance.

An additional consideration is the presence of potential outlier observations in some datasets. In such cases it is first sensible to validate the observation to ensure that no labelling error or data entry error has occurred. Assuming these explanations can be excluded, then it is prudent to re-calculate the FECR and 90 % CI both with and without the extreme individual to determine if the overall interpretation is affected by the potential outlier. In some cases, exclusion of these individuals may affect the overall classification, for example an inconclusive FECRT may become classified as resistant after exclusion, or vice versa. Unfortunately, there is no way to determine after the fact if the outlier value was a consequence of mis-dosing of the animal or mis-labelling of the sample, or if the result was a true biological outlier without a technical cause. Computational approaches have been developed to address this issue (Nielsen et al., 2013), but these are not easily applied and require the assistance of a qualified statistician. It is suggested that in these types of cases that the presence of the extreme individual be noted along with both sets of results and efficacy classifications. Alternatively, the test could be repeated.

3. Section 3: general guidelines for FECRT

In this section, general guidance is provided on (i) time since last anthelmintic treatment, (ii) handling of the samples, (iii) dosing of the animals, (iv) FEC process, (v) timing of follow-up samples, (vi) identifying parasite species, (vii) defining thresholds for expected efficacy and AR, and (viii) interpreting results of the FECRT.

3.1. Time since last anthelmintic treatment

Optimally, the FECRT should test the anthelmintic susceptibility of the overall suprapopulation of gastrointestinal nematodes on the farm (all parasites at all stages in their life cycle both within hosts and on the pasture), and should not be biased by recent anthelmintic treatments. If testing is performed too soon after the previous treatment, a substantial portion of the worm infrapopulation (subpopulation of parasites residing within the animals) may have undergone drug selection, and hence will not be representative of the overall worm suprapopulation on the farm. Thus, when an FECRT is performed, it is optimal that animals should not have been administered an anthelmintic for as long as possible prior to the test; however, a period of 8–12 weeks (Coles et al., 1992) will suffice for most commonly used anthelmintic products. If a long-acting product is used, one should wait at least 4–8 weeks past the label period of drug activity. These intervals should minimize the bias that the previous treatment might introduce. Note that these time frames assume animals are being continually reinfected from pasture. If reinfection is unlikely due to the existing environmental and/or housing conditions, then the test should be postponed until a later date. In addition, all animals in a test group should have a common history of housing/pasture and optimally should be of the same age class (juvenile vs. adult). Other host-specific and parasite-specific recommendations are provided in Section 4.

3.2. Sample handling

Faecal samples should be collected from each animal directly from the rectum, or fresh samples from the ground when the identification of the animal is certain. For horses kept in individual stalls, samples less than 12 h old are adequate (Nielsen et al., 2010b). Samples must be placed in individual sealed containers, kept cool during transport, and returned rapidly to the laboratory for refrigerated storage and/or for egg counting. Excluding air from the sample container (or vacuum packing) will prevent the development of strongyle eggs with or without refrigeration depending on species (Hunt and Taylor, 1989; Nielsen et al., 2010b; Sengupta et al., 2016). Samples should be protected from freezing or from reaching high temperatures (>40 ^{o}C) even for short periods of time, as either will decrease egg recovery (Nielsen et al., 2010b; Rinaldi et al., 2011). It is suggested that prior to removing an aliquot of faeces for the egg count, the faeces should be thoroughly broken up and/or mixed to create a more homogenous mixture of the entire sample that was collected. Faecal samples that will also be used for coproculture or for other in vitro tests may have additional requirements for collection, transport and storage, as cold storage for more than one day may adversely affect larval development, and this effect varies between different parasite species (McKenna, 1998). Lastly, for a given farm or stable, both the pre- and post-treatment samples should be collected and stored in exactly the same manner (or as closely as possible), and nutritional management of animals should remain consistent throughout the test period to reduce the potential for changes in faecal moisture.

3.3. Animal dosing

Optimally, animals should be weighed individually using a calibrated weight scale, and an appropriate dose based on the individual animal weight is administered as per product label instructions. If a scale is not available, a weight tape may be used for those animal species, for which calibrated weight tapes exist (e.g. cattle, horses, pigs and dairy goats). However, dosing based on individual weight may not be practical or possible in some instances. In such cases, the dose should be assigned to the group based on the dose required for the heaviest animal; however, this is only justifiable if the group is similar in weight, i.e. all animals are within approximately 20 % of the heaviest. Consequently, this approach can only be used when animals are fairly uniform in age and

breed. Dosing to the mean weight of a group should never be done, as this will result in approximately half of the animals being under-dosed. Finally, product expiration dates, and label recommendations for product storage, dosage and application technique should be followed closely, and dosing equipment should be calibrated and checked to ensure it is operating properly.

3.4. Requirements concerning performance of FEC

If a FEC is not available from an animal at both the pre-treatment and the post-treatment samplings, then the animal should be omitted from the data set. To increase the diagnostic power of the FECRT result, the total number of eggs counted pre-treatment under the microscope (the cumulative raw count of eggs before applying a conversion factor) across the group of animals should exceed the levels listed for the specific guidance (animal/drug/parasite and research/clinical) being followed (see Section 5). If fewer eggs are counted pre-treatment than are required, then a second FEC (or an additional chamber of the counting slide) from each animal should be counted until the egg tally exceeds the required level. Note that every animal will need to have the same number of FEC performed (total slides/chambers read). The post-treatment FEC should be measured using the exact same method and the same number of slides/chambers should be read as for the pre-treatment counts.

3.5. Timing of follow-up sampling

The post-treatment sampling interval needs to be long enough to allow the complete expulsion of eggs following the death of female worms and the resumption of normal egg production in surviving female worms, but short enough to prevent the development of newly established patent infections. A post-treatment interval of 10–14 days has been a standard recommendation for many years (Coles et al., 1992). For sheep, more specific intervals of 3–7, 8–10 and 14–17 days were recommended for levamisole (an IT), BZ and ML drugs, respectively, with 14 days recommended as the target interval when more than one anthelmintic class is being tested (Coles et al., 2006). The shorter time interval for levamisole was suggested due to the reduced efficacy levamisole demonstrates against immature and tissue-stage worms. A 10–14 day interval remains valid for sheep, horses and cattle treated with the short-acting drugs, with 14 days recommended for ML drugs. However, this interval is not optimal for cattle treated with ML drugs, which require a longer time interval. Post-treatment interval periods for cattle that vary from this 10–14 day recommendation are provided in Table 1 and explanations for these differences are explained in cattle-specific portion in Section 4.1.4.

Table 1
Recommended intervals between treatment and post-treatment faecal collections when performing FECRT on cattle.

Anthelmintic drugs	Interval (days)
Non-persistent drugs (e.g. levamisole, benzimidazoles)	10–14
Macrocyclic lactone drugs Avermectin drugs (e.g. ivermectin, doramectin, eprinomectin)	14–17
Moxidectin	17–21
Specially formulated long-acting macrocyclic lactone products	21–28
Both non-persistent and macrocyclic lactone drugs tested independently in the same experiment	Multiple post-treatment time points are optimal
If testing multiple drugs and only a single post-treatment time point is possible[a]	14
Drug combinations	Use the longest time interval recommended for an individual drug of the combination, or check at multiple timepoints post-treatment

[a] efficacy of moxidectin may be overestimated if tested at 14 days post-treatment.

3.6. Identification of species

Optimally, tests should be performed to differentiate the genera and/or species of the eggs present in the samples since infections are almost always composed of multiple species. Preferably this should be performed both pre- and post-treatment, to determine the relative changes due to treatment, and to determine which species likely are resistant and which likely are susceptible. Coprocultures have historically been the primary means to achieve differentiation of strongylid genera/species, however new molecular tests such as multiplexed tandem PCR (Roeber et al., 2017a; Roeber et al., 2017b) and the nemabiome assay (Avramenko et al., 2015) offer many benefits and could serve as useful alternatives if these were to become readily available as a diagnostic service (see Box 2). Pre- and post-treatment genera/species differentiation results can also be used to estimate the FECR for each genus or species, however, for the following reasons, such data should be interpreted carefully: (i) many factors can affect the development and recovery of various nematode species in culture, although this problem is mostly eliminated when using properly validated molecular methods for identification of parasite stages not requiring coproculture such as eggs or L1 larvae, and (ii) depending on the total number of eggs counted in the pre-treatment samples, there may be inadequate statistical power to accurately assign a resistance classification to individual genera or species. This issue also applies to *Nematodirus* spp.; though eggs are easily differentiated, EPG are typically too low to count sufficient numbers of eggs. In order to gain the required power to assign genera/species-specific classifications, many more eggs therefore need to be counted; the requirement for the number of eggs counted for each species would be the same as indicated in the general guidance without respect to genera or species. However, even if a sufficient number of eggs are counted, determining species-specific efficacy with appropriate CIs presents statistical problems that require additional research. This is because species identification is typically performed at the group level, whereas CIs for the FEC reduction are derived from the individual-animal FEC data. It is therefore not possible to generate species-specific CIs without making assumptions regarding the variation in relative species abundance between individual animals. Nevertheless, performing larval identifications and quantification in the pre- and post-treatment samples, particularly using newer sequencing methods, will allow one to determine the relative changes in genera/species intensity due to treatment, and based on this, inferences can be made regarding which species may be resistant and which are not. Even if not precise, this information has important clinical implications (see Box 2 for further insights on this issue).

4. Section 4: host-specific FECRT guidelines

The general biological and statistical issues relevant to the performance of the FECRT presented in Sections 2 and 3 are consistent across host animal and parasite species. However, there are a number of important host-specific, and in some cases, parasite species-specific issues that must be considered. While the differences among ruminant hosts are relatively minor, there are some major differences for horses and swine that are noteworthy. In this section, we discuss these issues and provide specific guidance for performing the FECRT in each host, and where relevant, for particular parasite species. The minimum group size and number of counted eggs required for each host species (and parasite type) are presented in Section 5.

4.1. Cattle

There are several cattle-specific issues in the experimental design of

Box 2
Molecular approaches to the relative quantitation of parasite genera or species abundance as an adjunct to the FECRT.

The total strongylid egg count often comprises one or more resistant and multiple susceptible nematode genera/species that are not readily distinguishable by microscopy; this reduces the diagnostic sensitivity of the FECRT. Further, depending on the spectrum of activity of the drug, a lack of genera/species-specific information makes the diagnosis of AR by the standard FECRT impossible, e.g. closantel resistance in sheep in regions where multiple trichostrongylid nematode species occur.

Quantitation of the relative abundance of each individual parasite species, in both pre-treatment and post-treatment faecal samples, provides an additional layer of information to help improve diagnostic sensitivity and confirm, or bring into question, the diagnosis of AR. The traditional approach used to quantify nematode species composition in faecal samples is morphological examination of third stage larvae (L3) recovered from coprocultures. This has major limitations including the long period of time required for development to L3, variance in development rate/mortality between faecal samples or nematode species, ambiguity of identification below the genus level, and the significant time and specialist expertise required.

PCR tests to identify individual strongylid nematode species have been available for many years. The internal transcribed spacer (ITS-2) rDNA is the most common target for this purpose, due to it having a level of sequence variation appropriate for unequivocal discrimination of many of the relevant nematode species in ruminants and horses (Hoste et al., 1995; Poissant et al., 2021). Despite the availability of PCR testing, routine use in conjunction with FECRT is uncommon. This is primarily due to the inability of standard PCR assays to provide quantitative species abundance data, as well as challenges with scalability, lack of comprehensive species coverage and cost. However, recent technical developments including multiplex real-time PCR assays (Roeber et al., 2015) and nemabiome metabarcoding (Avramenko et al., 2015; Redman et al., 2019) are now overcoming these challenges, making it increasingly feasible to integrate molecular-based species abundance data with FECRT data (Queiroz et al., 2020). Nemabiome metabarcoding involves next-generation sequencing of ITS-2 rDNA amplicons for strongylid nematode species identification and relative quantitation (Avramenko et al., 2015) (https://www.nemabiome.ca/). This method generates thousands to millions of ITS-2 rDNA sequence reads per sample that are mapped against a reference database to provide accurate and repeatable species identification of hundreds or thousands of eggs or larvae in a sample as required. Nemabiome metabarcoding dispenses with the need for species-specific primers and individual species assay optimization. A comprehensive, curated and regularly updated ITS-2 rDNA database is now available increasing the reliability of species identification including those unanticipated to be present in a sample (Workentine et al., 2020). To date, the method, which can be applied to eggs, L1s or L3s, has been applied to cattle, sheep, bison and horses (Avramenko et al., 2017; Avramenko et al., 2018; Redman et al., 2019; Poissant et al., 2021). Current capabilities and practical issues including the choice of parasite stage have been recently reviewed in some detail (Redman et al., 2019; Kotze et al., 2020). The ability to multiplex a large number of samples on a single sequencing run currently makes it most cost-effective when batching large numbers of samples although the current rapid development of sequencing technologies makes increased flexibility and availability soon likely (Kotze et al., 2020).

Species abundance data from multiplex real-time PCR or nemabiome sequencing can be used to convert total FEC data into interpolated species-specific egg counts (Queiroz et al., 2020). However, one should keep in mind that following conversion of total FEC data into interpolated species-specific egg counts, the required numbers of counted eggs provided in Section 5 will continue to apply to each separate species for which a species-specific diagnosis is desired.

The value of species-specific information for the interpretation of FECRT includes:

Determination of the number and identity of the AR parasite species, enabling more evidence-based decisions regarding clinical impacts, targeted drug choice and subsequent management.

Increased sensitivity of the FECRT to detect AR of specific genera/species in the presence of multiple co-infecting susceptible species.

Detection of AR to narrow spectrum anthelmintic drugs, which is otherwise impossible using total strongyle egg count data alone.

Determination of changes in parasite species diversity following anthelmintic treatment to help confirm, or question, a diagnosis of AR.

an FECRT that require additional consideration, including (i) sampling strategy, (ii) age of the animals, (iii) drug formulation, (iv) dosing of the animals, and (v) follow-up period.

4.1.1. Sampling strategy

In these guidelines, it is recommended that the same animals be tested both pre- and post-treatment. The time and effort required to run cattle through a chute makes it tempting to take random samples post-treatment, but this should be avoided. Due to the high level of variability in FECs among cattle in a herd, sampling different animals pre- and post-treatment creates statistical issues that makes it difficult to estimate the efficacy with accuracy, leading to rather wide CIs. Consequently, this approach is not recommended in these guidelines, particularly for research purposes. However, in a clinical setting, if one is willing to accept a potentially larger inconclusive (grey) zone and can perform the analysis correctly by taking into account the non-standard study design, then such a test would be possible. The use of a non-paired sample strategy is not addressed in this guideline and one should consult with a qualified statistician if that approach is used.

4.1.2. Age of the animals

If possible, the FECRT should be done only on cattle less than 16 months of age, since the low mean FECs of adult cattle make it difficult to count the numbers of eggs necessary for a high diagnostic performance of the FECRT to detect AR. In situations where a test is desired on adult cattle, FECRT will require additional modifications. Large numbers of animals and/or a FEC method with a very low multiplication factor will be necessary. Even with these modifications, it may still be difficult to achieve sufficient egg counts for a reliable result, and in such cases, it may be necessary to perform two or more FECs on each animal.

4.1.3. Formulation

The pharmacokinetics of an anthelmintic drug involves the time course of drug absorption, distribution, metabolism and elimination from the host, which, in turn, determines the concentration of the active drug that the parasite is exposed to (Lanusse et al., 2014). Consequently, the route of drug administration may have a marked impact on the drug response, but these differences on drug efficacy attributed to the administration route may only be evidenced if the parasite population has a reduced susceptibility (Lanusse et al., 2014). Because of these

pharmacokinetic issues and the multiple routes that are available for administration, when performing an FECRT with ML drugs, an additional consideration is the purpose of the test. If the purpose is to determine whether ML resistance is present, such as in an AR prevalence survey, then it is generally preferable to use oral or injectable formulations. In contrast, pour-on formulations may yield reduced efficacies (therapeutic failures) due to poor/erratic drug absorption that are not necessarily caused by resistance to the drug. There is clear evidence for the erratic percutaneous absorption of topically administered ML in cattle in addition to the high variability in systemic exposure between animals, due to both allo- and self-licking (Laffont et al., 2001; Bousquet-Melou et al., 2004). In addition, haircoat type and length, soiling of haircoat and poor application technique can all lead to reduced efficacies not related to AR (Sargison et al., 2009). Nevertheless, in many parts of the world, topical (pour-on) products remain the most commonly used application method for anthelmintic administration to cattle, and if the goal is to determine whether the pour-on treatments being used on a given farm are providing effective parasite control, then it is appropriate to use pour-on products in an FECRT. However, the use of pour-on formulations requires additional modifications in experimental design, and when reduced efficacies are observed, results should be interpreted cautiously with regard to declaring AR. Ideally, any test with a pour-on that indicates AR, particularly when results are close to the thresholds used for declaring resistance, should be repeated using an oral or injectable formulation of the same ML molecule before declaring AR being present on that farm. Foremost in experimental designs based on pour-ons, it is recommended to treat all in-contact cattle on the pasture with the same product, and if other formulations are tested at the same time then animals in those groups optimally should be physically separated since allo-licking is typical of bovine natural behaviour and may cause drug transfer between animals. Therefore, the impact of licking behaviour should be considered as a biological variable in the design of efficacy studies when using topically applied anthelmintics (Toutain et al., 2012).

4.1.4. Timing of follow-up sampling

As mentioned in the general section, the post-treatment sampling interval needs to be long enough to allow the complete expulsion of eggs following the death of female worms and the resumption of normal egg production in surviving female worms, but short enough to prevent the development of newly established patent infections. *Cooperia* spp. have the shortest pre-patent period of all the strongylid nematodes of cattle at 11–13 days (Leland, 1995). For other strongylid species of cattle the minimum pre-patent period is 18 days (excluding *Nematodirus* spp. whose eggs are easily distinguished) (Wood et al., 1995). However, these published pre-patent periods were measured in studies where first inoculations were administered to parasite naïve calves. After second and third inoculations, the pre-patent periods of *Cooperia* spp. were significantly ($p < 0.02$) longer, averaging 19.6 and 23.4 days, respectively (Leland, 1995). These data suggest that though it is possible by day 14 for *Cooperia* spp. eggs to appear in the faeces of treated calves that are derived from larvae ingested after treatment, the likelihood of this seems quite low under field conditions. This is particularly true for the ML drugs, all of which have persistent activity against nematodes susceptible to these drugs. This assumption is further supported by data from field studies where cattle treated with injectable formulations of either abamectin, doramectin, ivermectin or moxidectin and grazed on contaminated pastures demonstrated a greater than 99 % reduction in FEC for at least 21 days (Entrocasso et al., 1996; Meeus et al., 1997).

On the other hand, temporary egg suppression in female worms has been reported on numerous occasions in both sheep and cattle (Watson et al., 1996; Sutherland et al., 1999; Condi et al., 2009). In sheep treated with BZ, this suppression is short-lived and does not appear to impact the FECRT (McKenna, 1997). However, in cattle treated with ivermectin there is evidence that a 10-day interval is too short and a 14-day interval may be preferable (McKenna, 1997). Additionally, with moxidectin, egg suppression may last for more than 14 days post-treatment. Condi et al. (2009) reported that 98.5 % of *Cooperia* spp. females recovered from control animals at necropsy had eggs inside the uterus, as compared to only 48.2 % of the females recovered from the moxidectin-treated group. Similarly, De Graef et al. (2012) reported 43 % less eggs in adult female *C. oncophora* recovered from moxidectin-treated calves as compared to control calves that were necropsied 14- or 15-days post-treatment. Thus, when moxidectin is used in an FECRT, a post-treatment interval of 14 days may not be sufficient, and a 17–21 day interval preferred (Kaplan and Vidyashankar, 2012).

Unfortunately, there is no easy solution for this conflict between life cycle length and egg suppression period, particularly following moxidectin treatment. Many poorly understood factors contribute to variability in both parameters. However, given available evidence, sound recommendations for the post-treatment interval can be developed (see Table 1). With non-persistent drugs such as BZ or levamisole, it is recommended that post-treatment sampling be performed at 10 days, with 10–14 days being acceptable. For avermectin drugs, it is recommended that post-treatment sampling be performed at 14–17 days and for moxidectin at 17–21 days. For specially formulated long-acting ML drugs there is insufficient data to define the optimal time point, and this could vary based on the specific drug and formulation. However, a longer interval should be used for long-acting products and a timeframe of 21–28 days seems most appropriate. When both ML and non-ML drugs are tested independently at the same time on the same farm, multiple post-treatment time points as recommended here would be optimal. When multiple post-treatment faecal collections are not possible, then a 14-day interval would be the preferred compromise time given the various factors that must be considered. When testing combination products or multiple anthelmintics given concurrently in combination, then the longest time interval recommended for an individual drug of the combination should be used.

4.2. Small ruminants

4.2.1. Sheep

The general recommendations provided in Sections 2 and 3 apply well to sheep, and this is probably true more so than for any of the other host animal groups. Thus, there are few host-specific issues that need to be addressed. The only major issue that deserves a brief discussion is the use of a non-paired strategy (with a non-treated control group). In commercial sheep enterprises, at suitable times, many young sheep with a relatively high FEC are available to conduct an FECRT, and this may often involve testing a number of different anthelmintics as well as drug combinations in the same test (Waghorn et al., 2006; Playford et al., 2014). Although the non-paired approach can be more practically feasible than the paired strategy when more than three products are to be tested, this is true only if sampling is performed just once at the post-treatment period. However, most large studies utilizing a non-paired strategy still perform both pre- and post-treatment FEC (Rendell et al., 2006; Waghorn et al., 2006; Falzon et al., 2013; Mederos et al., 2014). This then negates any logistical benefits of the non-paired strategy, and as discussed in Section 2.1, the paired strategy yields narrower CIs. Thus, the paired strategy as recommended in this document fully applies to small ruminants as well.

4.2.2. Goats

Few anthelmintic products have label approval for goats and so treatment of goats is almost always performed in an extra-label fashion. Additionally, goats metabolize anthelmintic drugs differently than sheep (Scott et al., 1990; Sundlof and Whitlock, 1992; Escudero et al., 1999; Chartier et al., 2000; González Canga et al., 2009), and it is generally recommended that goats be administered 1.5 times the sheep dose of levamisole (Chartier et al., 2000; Hoste et al., 2011) and 1.5–2 times the sheep dose for other anthelmintics (Hoste et al., 2011). If the recommended sheep dose of anthelmintic is used in goats, it may

produce a reduced efficacy unrelated to AR. Thus, any test for AR using FECRT in goats must be performed using extra-label dosages.

4.3. Horses

In this section, horse-specific guidelines are provided for both cyathostomins (small strongyles) and *Parascaris* spp. Other nematode parasites of horses are not addressed in these guidelines. To date there is no credible evidence that AR has occurred in *Strongylus* spp. (large strongyles) and numbers of large strongyle eggs shed by horses are too small to draw reliable conclusions on the efficacy of the administered drugs using an FECRT. The AR status of other non-strongyle parasites of horses either are not conducive to evaluation by FECRT or they are not readily detectable by FEC (i.e. *Oxyuris equi, Anoplocephala perfoliata*), and therefore are not addressed in this document.

4.3.1. Cyathostomins

Performing FECRT on horse farms/stables often presents several additional challenges not commonly encountered in ruminant operations (Vidyashankar et al., 2012). The most important of these are the availability of few horses to test, very low FECs in many horses, high variability of FEC even from within the same horse (Denwood et al., 2012), and a highly heterogeneous population with regard to age, sex and breed. Furthermore, 40 cyathostomin species infecting horses have been described (Lichtenfels et al., 2008), with 10–20 species commonly encountered in naturally infected horses (Bellaw and Nielsen, 2020). Also different from ruminants, coprocultures have limited value in equine FECRT studies, as the morphology of L3 larvae does not allow differentiation of cyathostomins to genera/species. Finally, the expected efficacies in drug-susceptible populations of cyathostomins differ for the three major anthelmintic families; therefore, uniform criteria for interpreting FECRT results should not be used. It is particularly important to note that the difference in expected efficacies for MLs and the IT, pyrantel (ML > pyrantel) lead to the requirement for much larger sample sizes for assessing the efficacy of pyrantel as compared to that for MLs.

4.3.1.1. Age of the horses. The magnitude of pre-treatment strongylid egg counts differs between young (less than 2 years old) and mature horses (Herd and Gabel, 1990; Herd and Majewski, 1994), although there is no strong evidence to suggest that this leads to different efficacy estimates between age groups (Drudge et al., 1982; Boersema et al., 1996). Nonetheless, if more than one drug is being tested, a randomized block design should be employed, wherein horses are first categorized into age groups; yearlings, 2–4, 5–15, and > 15 years. It is recommended that foals not be used for determining drug efficacy for cyathostomins, given their often lower and variable egg count magnitude and an apparently different progression of the cyathostomin life cycle (Nielsen and Lyons, 2017). Then, rank horses within each age group according to decreasing magnitude of strongylid raw FEC, randomly assign them into blocks corresponding to the number of desired treatment groups, and then within a block, randomly allocate them to group using a random number generator.

4.3.1.2. Sample collection. On horse farms, faeces are often collected from the stall floor, whereas for ruminants, faeces are most often collected directly from the rectum. A study conducted in the USA and Denmark demonstrated no significant differences in FECs when samples collected from horse stalls were 12 h old or fresher (Nielsen et al., 2010b), and there is no evidence of diurnal variation in FEC of horses (Denwood et al., 2012; Carstensen et al., 2013). Thus, when horses are stabled singly, collecting the freshest sample available from the stall floor will provide a quality sample for FECRT. The sample should be free of debris, preferably collected from the centre of the dung pile. Additionally, unlike some of the species of ruminant parasites, viability of equine strongyle eggs is not impacted by cold storage, although coprocultures have limited value in equine studies as outlined above. Thus, cold (4 °C; but not freezing) storage is always recommended. Though it is suggested that FEC be performed on fresh samples, no significant decrease in strongylid EPG was detected after 5 days of refrigeration in airtight containers (Nielsen et al., 2010b; Sengupta et al., 2016).

4.3.1.3. Dosing. The difficulty with precision in dosing is another technical factor to consider when performing FECRT in horses. It is uncommon for horse farms to have scales for weighing horses, thus weight tapes are commonly used to determine the weight of horses. However, this measurement only provides an estimate, as there are many conformational, breed and physiological factors that cause error in this measurement, and there is potential for user error. Additionally, dose calibration marks on tubes of anthelmintic may not be precise depending on the product, as calibration line intervals vary from 25 to greater than100 kg. Consequently, when weight tapes are used, it is recommended that a dosage equal to the weight tape estimation plus 15–20 % is administered to reduce the chances of under-dosing. Furthermore, when performing FECRTs for research and/or regulatory purposes, it is also recommended that the tube of anthelmintic paste (gel) be weighed both before and after administration to confirm the actual amount of anthelmintic that was delivered. Alternatively, one could empty the tube and transfer the measured dose to a translucent syringe for administration.

4.3.1.4. Drug-specific issues and thresholds for evaluating FECR data. Perhaps the most important equine-specific difference is that each of the three major families of anthelmintics used in horses differs in their expected efficacies against susceptible cyathostomin populations (Kaplan, 2002). Consequently, different criteria for sample size, numbers of eggs counted, and interpretation of results must be used for evaluating each drug class. Following treatment with a ML (either ivermectin or moxidectin), expected FECR is 99.9 %, and in most horses, no eggs are seen in the post-treatment FEC (Boersema et al., 1991; Kaplan et al., 2004; Stratford et al., 2013; Smith et al., 2015). Following treatment with BZ, FEC reduction in the early studies was often, but not always 100 %, thus expected reductions in FEC for BZ are 99 % (Drudge et al., 1975, 1978., 1979; Malan and Reinecke, 1980; Malan et al., 1981). In contrast, following treatment with tetrahydropyrimidines (pyrantel, morantel), FEC reductions are more variable, ranging from 96 % to 100 % (Lyons et al., 1974; Drudge et al., 1982). A review of pyrantel efficacy data in early studies (1975–78) included more than 100 horses across 10 separate studies, including weanlings, yearlings, mares and stallions (Drudge et al., 1982). The mean efficacy of those studies was 98.2 % and excluding studies that may have had a biased result due to mean pre-treatment FEC less than 250 EPG, the mean percent reduction was 98.7 %. Another study that included almost 200 horses divided among three trials in Kentucky and North Carolina reported FEC reductions of 99 %, 98 % and 98 % for pyrantel paste (Newby et al., 1982). Thus, it seems reasonable to assume an expected efficacy for pyrantel of 98 %. Though these differences in expected efficacies between drug classes may seem small, they have a major impact on the amount of variability that can be expected in the resulting FECRT data. This higher level of variability will have an important impact on the ability to accurately detect a reduction in efficacy (Levecke et al., 2015; Denwood et al., 2023).

With regard to diagnosing resistance to MLs in cyathostomins, there are some additional issues that make this exercise slightly different than for the ruminants or for the other equine drugs. The percentage reduction in FEC for susceptible populations of equine strongyles is extremely high for the MLs, with no eggs being seen post-treatment in the majority of horses; consequently, criteria for diagnosing AR should be more stringent than for other equine or ruminant anthelmintic drugs. Additionally, since ML resistance in cyathostomins appears to still be rare, it

is advisable to repeat any test yielding reduced efficacy to confirm the initial result (Nielsen et al., 2020). This is particularly important in large surveys, as the chances one will detect a farm with reduced efficacy that is not due to AR increases as more farms are tested (Vidyashankar et al., 2012). This principle is not specific to MLs in horses; this recommendation should be followed for any drug-parasite combination where resistance prevalence is very low or has not been reported previously. Another issue that can arise in an FECRT in horses treated with ML drugs is the possibility of observing 100 % efficacy in all horses, yet still having an inconclusive result based on the lower CI. Note that this will not occur when numbers for group size and eggs counted follows the guidance provided in Section 5. However, if few horses are available to test, and/or mean FECs are too low to reach the required number of counted eggs, such an outcome is quite possible. Nevertheless, if the FECRT is performed for routine farm-level monitoring purposes, it would be reasonable to assume that AR is unlikely.

Several reports of shortened egg reappearance periods (ERPs) following ML treatment have been published in recent years, and these are generally being interpreted as evidence of emerging resistance to this drug class (Peregrine et al., 2014; Relf et al., 2014; Molena et al., 2018). However, recent investigations do not support the suggestion that shortened ERPs are signs of emerging anthelmintic resistance (Nielsen et al., 2022). Thus, the interpretation of shortened ERPs is not clear, and they cannot be solely regarded as evidence of emerging anthelmintic resistance (Nielsen, 2022). Nevertheless, this development is a substantial change of drug performance from previous years with obvious implications for strongyle control. Thus, monitoring of ML ERPs has practical value for veterinarians and their clients, but at this point should not be viewed as a direct indicator of emerging AR.

4.3.1.5. Sample size. The required number of horses depends on a number of different factors as discussed in previous sections of this document and these are presented in Section 5. However, whereas most ruminant farms will have sufficient animals present to meet the guideline parameters, many horse farms will have only a few horses, and it is likely that some of those horses will not have detectable levels of strongyle eggs. Consequently, an FECRT must often be performed with fewer than the recommended number of horses. Because of this limitation, it is advised that horses be selected for inclusion based on their individual FEC. This can be done by performing a screening FEC on each horse prior to the test, or alternatively, using historical FEC surveillance data, since FECs of horses tends to be relatively consistent over time (Eysker et al., 2006; Nielsen et al., 2006). Additionally, pre-test EPGs should be considered when selecting a FEC method to maximize the likelihood that the necessary number of counted eggs is achieved. Given the possibility that the numbers of horses required in the guidelines may not be available to test, when performing an FECRT for research purposes one should test as many horses as possible, and never fewer than six horses. In a clinical situation, testing fewer horses than recommended does not mean the results have no utility; it just means that a definitive result becomes less likely, and the results need to be interpreted more carefully. Though an accurate classification may not be possible because of wide CIs, when efficacy is very high (e.g. >98 %) or very low (e.g. <80 %), a useful clinical inference may still be possible.

4.3.2. Parascaris spp

The equine ascarid has traditionally been referred to as *Parascaris equorum*. However, two *Parascaris* species infect horses, and recent work suggests that *P. univalens* may be the predominant species in managed horses (Nielsen et al., 2014; Martin et al., 2018). Unless karyotyping by visualizing and counting the chromosomes is performed (Martin et al., 2018), it is most appropriate to refer to the parasite genus, i.e. *Parascaris* spp.

In contrast to cyathostomins, all anthelmintic drug classes originally demonstrated similar very high efficacy against adult *Parascaris* spp. with no eggs seen post-treatment (Lyons et al., 1974, 1992; Drudge et al., 1975, 1978,1979, 1984; Klei and Torbert, 1980; Kingsbury and Reid, 1981; French et al., 1988; Čorba et al., 1995). Thus, for the purposes of this guideline, the expected efficacy for all three major anthelmintic classes against adult *Parascaris* spp. is set as 99.9 %.

The evaluation of drug efficacy against *Parascaris* spp. by FECRT is affected by several factors, most important of which are the strong age-related immune response and the potential for density-dependent alterations in female worm fecundity (Clayton and Duncan, 1977). In general, *Parascaris* spp. egg shedding is tightly restricted by foal age with a majority of foals being egg count positive between 3 and 6 months of age, after which the worms are most often eliminated (Donoghue et al., 2015; Fabiani et al., 2016). During this time window, ascarid egg counts tend to increase, reaching a peak at about 4–5 months of age, after which they decline and eventually become negative (Donoghue et al., 2015; Fabiani et al., 2016). Thus, foal age can confound the FECRT estimate; in a study where resistance to ivermectin was documented, the efficacy was significantly higher in older foals (Craig et al., 2007).

An additional issue that can confound an FECRT is coprophagy, which has the potential to cause false positive ascarid FEC results in foals. The false positive rate for *Parascaris* spp. egg counts has been reported as 6 % for horses predominantly less than 2 years of age (Nielsen et al., 2010a). Most of these false positives were less than 100 EPG, with only ~1 % of all false positive foals with greater than 100 EPG. This suggests false positives due to coprophagy are not a major problem; however, given this potential, only foals with a minimum of 100 *Parascaris* spp. EPG) should be enrolled in an FECRT. Thus, a screening FEC should be performed prior to performing a *Parascaris* spp. FECRT on foals.

4.3.2.1. Experimental design – research protocol. In most equine operations, foals are typically born over the course of several months leading to a range of ages represented at any proposed treatment date. This means that most often there will an insufficient number of similarly-aged foals on a farm to perform a one-time FECRT. To address this issue, a recent study demonstrated the principle of rolling enrolment, where foals were monitored over the course of 3 months and assigned randomly to treatment groups as they met the inclusion criterion of being ascarid egg count positive (Morris et al., 2019). This ensured a more uniform age and ascarid FEC profile for the foals receiving anthelmintic treatment, and likely increased the number of foals eligible for the study. As a general guidance, foals should be in the range of 4–8 months old, corresponding to the age at which they are most likely to be shedding ascarid eggs, but eligibility for inclusion should ultimately be determined by a positive ascarid egg count.

Given these issues, the following study design is recommended. A rolling enrolment scheme should be used, except for the rare situations when a sufficient number of foals of similar age are available for testing. Starting at 3 months of age, foals are monitored for ascarid egg shedding on a monthly basis. Each month, those foals with a pre-treatment screening FEC greater than 100 EPG are blocked by age-month, and then within each block foals are assigned randomly to either a non-treated control or treatment group. If blocks of age-matched foals have an odd number, then the extra foal should be placed in the treated-foal group. All foals should have a pre-treatment FEC performed on the day of treatment and another FEC 10–14 days after treatment. Though not required, performing two or three independent pre- and post-treatment FEC on each foal and using the sum of the FEC will reduce variability and therefore increase the chances of a non-inconclusive result. For performing the FECRT analysis, only the pre- and post-treatment FEC of the treated group are used; the screening FEC data are discarded and the FEC of the non-treated control foals are used only to monitor natural changes in the *Parascaris* spp. FEC. If FEC in the control group spontaneously reduce by more than would be expected by random chance (given the observed mean and variability in FEC), then

the FECRT data recorded in the treatment group should be interpreted with great caution.

4.3.2.2. Experimental design – clinical protocol. All foals available for treatment should be included in the FECRT. A pre-treatment screening FEC can be performed and only those foals with *Parascaris* spp. FEC greater than 100 EPG included in the test. Alternatively, the screening FEC can be omitted and the data from any foals with a pre-treatment FEC less than 100 EPG are excluded from the analysis. This study design makes performing an FECRT for clinical purposes more practical on more farms, however, it should be appreciated that the results may be confounded by the issues outlined above. When using the clinical protocol for *Parascaris* spp., these additional complicating factors make the interpretation of FECR data more uncertain as compared to when using the clinical protocol for cyathostomins or other host species.

4.4. Pigs

The most important nematodes in pigs in temperate and subtropical climates include *Ascaris suum*, *Trichuris suis* and several species of *Oesophagostomum*. There is evidence of AR in *Oesophagostomum* spp. against pyrantel, levamisole, BZ (flubendazole) and ivermectin; all confirmed under controlled conditions (Roepstorff et al., 1987; Bjorn et al., 1990; Bauer and Gerwert, 2002; Gerwert et al., 2002; Macrelli et al., 2019). At present, there is no credible evidence of AR in any other species, but fewer surveys of AR have been performed in pigs compared to other host species. Most anthelmintics have notoriously poor efficacy against *T. suis*; levamisole and fenbendazole need to be used at increased doses or by repeated dosing over 3–5 days (Batte, 1978; Marti et al., 1978), and the efficacy of ivermectin against *T. suis* in the original studies was also low (80 %) (Benz et al., 1989). Whether this poor efficacy is related to inadequate drug uptake (Hansen et al., 2014), low penetration of drugs locally in the caecum and colon, an inherent or acquired low susceptibility at molecular level (Diawara et al., 2013) or some other aspect of worm biology remains unknown.

In *A. suum*, appropriate calculation of CIs for egg count reductions is frequently complicated by extremely high variance of egg counts, coupled with occasional observations of high post-treatment egg counts in some animals of groups, which otherwise demonstrate observed egg count reductions of 100 % (Roepstorff et al., 1997; Boes et al., 1998). Furthermore, false positive faecal samples are commonly seen for *A. suum*: a review of 12 controlled studies revealed that 4–36 % of *A. suum* faecal positive pigs had no intestinal worms, and false positive egg counts ranged from 20 to 1060 EPG, with the majority (85 %) of counts below 200 EPG (Boes et al., 1997). These issues make it difficult to evaluate drugs for resistance with the FECRT in both *T. suis* and *A. suum*, and to date there are no confirmed cases of AR in either parasite. Therefore, this guideline does not provide specific FECRT methods for these parasites at present. In contrast, *Oesophagostomum* spp. do not share these same confounding issues. Nevertheless, there remains several pig-specific issues that should be considered when performing an FECRT with *Oesophagostomum* spp.

4.4.1. Biological experimental design considerations for Oesophagostomum spp

The prevalence of *Oesophagostomum* spp. infection within a herd tends to be variable and is often quite low (Roepstorff et al., 1998; Haugegaard, 2010). Given these issues, an FECRT can only be reasonably performed on rather large groups of pigs (see Section 5). When performing an FECRT, it is recommended that pigs have not been treated with an anthelmintic drug within at least 2–3 months. Due to the potential for high levels of coprophagia (Sansom and Gleed, 1981; Soave and Brand, 1991), it is also recommended that all pigs in a pen (or enclosure) are treated with the anthelmintic drugs being tested, even if all animals are not sampled for the FECRT. One must also consider the ability to properly identify the parasites infecting the pigs. *Oesophagostomum* spp., *Hyostrongylus rubidus*, *Trichostrongylus axei* and *Globocephalus* spp. all have strongyle-type eggs that cannot be distinguished microscopically (Roepstorff and Nansen, 1998) and several different species of *Oesophagostomum* can infect pigs. This has relevance to the interpretation of the FECRT, as evidence suggests that ivermectin has lower efficacy in *O. quadrispinulatum* than in *O. dentatum* (Varady et al., 1996). Therefore, local knowledge of parasite occurrence and origin of samples (e.g. absence of *H. rubidus* in indoor systems), as well as parasite identification using larval coprocultures, PCR, or other molecular identification methods are recommended to interpret strongyle eggs counts and to select pigs for an FECRT. In line with the recommendations for other species, the exclusive use of paired study designs is advocated, with pre- and post-treatment samples taken from the same animals.

4.4.2. Threshold for evaluating FECRT data

The anthelmintics most commonly used against pig nematodes include MLs (ivermectin and doramectin) and BZs (fenbendazole and flubendazole), while levamisole, pyrantel and piperazine may also be available in some countries. Early efficacy studies (based on worm counts) of ivermectin using subcutaneous administration at 300 μg/kg yielded efficacies of 96 % and 95 % for adult and larval *Oesophagostomum* spp., respectively (Benz et al., 1989). However, later studies with ivermectin administered at the same dose using 'assumed' susceptible strains demonstrated efficacies against adult *O. dentatum* ranging from 69.1 % to 96.2 % (Petersen et al., 1996; Borgsteede et al., 2007). Notably, ivermectin appeared to be more effective against female worms than male worms in both of these studies, and in the one study where both worm counts and reductions in FEC were measured concurrently, the percent reductions in FECs were considerably higher than for worm counts, viz. 98.2 vs. 69.1 and 99.9 vs. 96.2 (Petersen et al., 1996). Additionally, worm reductions were considerably higher when measured at 14 days vs. at 6 or 7 days. With BZs, several studies on fenbendazole report reductions in adult worm burdens greater than 99.8 % when dosed orally at 6 mg/kg or 3 mg/kg for 3 days (Enigk et al., 1974; Marti et al., 1978; Stewart et al., 1981; Marchiondo and Szanto, 1987). Given this evidence, we therefore advocate for *O. dentatum* efficacy targets for FECR of 95 % and 99 % for ivermectin and BZs, respectively. There are insufficient efficacy data to make a recommendation for *O. quadrispinulatum*, so it is important to note that these guidelines refer only to *O. dentatum*.

5. Section 5: guideline for group size and required numbers of counted eggs

Contingent on the general and species-specific requirements outlined in the first three sections, this section provides the recommended group size for an FECRT with a given drug, host and parasite species. These guidance's are provided separately in Boxes 3–9. Furthermore, we provide two versions of guidance: (i) a more resource-intensive 'research protocol' that is intended for use in scientifically based studies, and (ii) a less demanding 'clinical protocol' requiring fewer animals and fewer eggs counted, which is generally intended for use by veterinarians and livestock owners. It should be reiterated here that both approaches can be considered equally robust from a statistical and scientific perspective. However, because of the inherent trade-off between logistical considerations and the expected sensitivity to detect small reductions in efficacy, the 'clinical protocol' has a wider 'grey zone', and thus will more often yield inconclusive results as compared to the 'research protocol'.

For each of these two protocols and for each species, we provide minimum group sizes to be included in the test, based on three different assumptions regarding the expected mean number of eggs counted in the pre-treatment samples. In general, if the FEC (in EPG) of the test group is unknown, then it is safer to use the version with more animals and to use a FEC method with a lower multiplication factor. In contrast, when the

Box 3
FECRT guidance for ruminants.

Target efficacy is 99 % for all anthelmintics.

Lower efficacy target is 90 % for the clinical protocol and 95 % for the research protocol.

This yields grey zones of 90–99 % and 95–99 % for the clinical and research protocols, respectively.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 90–99 %)	Research protocol (grey zone: 95–99 %)
Minimum mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	8 animals (Total eggs = 320)	15 animals (Total eggs = 600)
Minimum mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	11 animals (Total eggs = 165)	22 animals (Total eggs = 330)
Minimum mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	14 animals (Total eggs = 112)	31 animals (Total eggs = 248)

Box 4
FECRT guidance for cyathostomins in horses treated with macrocyclic lactones.

- Target efficacy is 99.9 %.
- Lower efficacy target is 92 % for the clinical protocol and 96 % for the research protocol.
  - o This yields grey zones of 92–99.9 % and 96–99.9 % for the clinical and research protocols, respectively.
- Note that resistance to ML drugs in cyathostomins continues to be uncommon, thus it is strongly recommended that all tests yielding a reduced efficacy are confirmed with a second test prior to declaring that ML resistance is present.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 92–99.9 %)	Research protocol (grey zone: 96–99.9 %)
Minimum mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	5 animals (Total eggs = 200)	7 animals (Total eggs = 280)
Minimum mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	7 animals (Total eggs = 105)	11 animals (Total eggs = 165)
Minimum mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	11 animals (Total eggs = 88)	17 animals (Total eggs = 136)

Box 5
FECRT guidance for cyathostomins in horses treated with benzimidazoles.

- Target efficacy is 99 %.
- Lower efficacy target is 90 % for the clinical protocol and 95 % for the research protocol.
  - o This yields grey zones of 90–99 % and 95–99 % for the clinical and research protocols, respectively.
- Note that resistance to BZs in cyathostomins is very common; if the true efficacy is far below the grey zone, then a conclusive result may be obtained using far fewer horses than is listed for this guideline.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 90–99 %)	Research protocol (grey zone: 95–99 %)
Minimum mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	7 animals(Total eggs = 280)	13 animals(Total eggs = 520)
Minimum mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	10 animals(Total eggs = 150)	19 animals(Total eggs = 285)
Minimum mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	13 animals(Total eggs = 104)	28 animals(Total eggs = 224)

Box 6
FECRT guidance for cyathostomins in horses treated with pyrantel.

- Target efficacy is 98 %.
- Lower efficacy target is 80 % for the clinical protocol and 88 % for the research protocol.
  - o This yields grey zones of 80–98 % and 88–98 % and for the clinical and research protocols, respectively.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 80–98 %)	Research protocol (grey zone: 88–98 %)
Mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	6 animals (Total eggs = 240)	9 animals (Total eggs = 360)
Mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	7 animals (Total eggs = 105)	11 animals (Total eggs = 165)
Mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	9 animals (Total eggs = 72)	15 animals (Total eggs = 120)

Box 7
FECRT guidance for *Parascaris* spp. in horse foals.

- Target efficacy is 99.9 % for all anthelmintics.
- Lower efficacy target is 90 % for the clinical protocol and 95 % for the research protocol.
  - o This yields grey zones of 90–99.9 % and 95–99.9 % for the clinical and research protocols, respectively.
- Note that in addition to the numbers provided here for the treated horses, we also recommend that an age-paired control group be included to gauge natural changes in the FEC; however, the control horse data is not used in the calculation of % FECR.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 90–99.9 %)	Research protocol (grey zone: 95–99.9 %)
Mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	5 animals (Total eggs = 200)	5 animals (Total eggs = 200)
Mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	6 animals (Total eggs = 90)	9 animals (Total eggs = 135)
Mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	8 animals (Total eggs = 64)	14 animals (Total eggs = 112)

Box 8
FECRT guidance for *Oesophagostomum dentatum* in pigs treated with benzimidazoles.

- Target efficacy is 99 %.
- Lower efficacy target is 88 % for the clinical protocol and 93 % for the research protocol.
  - o This yields grey zones of 88–99 % and 93–99 % for the clinical and research protocols, respectively.
- Note that this guidance is only applicable to *O. dentatum*. There are insufficient published data for drug efficacy to develop a guideline for *O. quadrispinulatum.*

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 88–99 %)	Research protocol (grey zone: 93–99 %)
Mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	5 animals (Total eggs = 200)	8 animals (Total eggs = 320)
Mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	7 animals (Total eggs = 105)	12 animals (Total eggs = 180)
Mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	10 animals (Total eggs = 80)	17 animals (Total eggs = 136)

Box 9
FECRT guidance for *Oesophagostomum dentatum* in pigs treated with ivermectin.

- Target efficacy is 95 %.
- Lower efficacy target is 80 % for the clinical protocol and 85 % for the research protocol.
  - o This yields grey zones of 80–95 % and 85–95 % for the clinical and research protocols, respectively.
- Note that this guidance is only applicable to *O. dentatum*. There are insufficient published data for drug efficacy to develop a guideline for *O. quadrispinulatum*.

Minimum mean number of eggs counted in pre-treatment samples vs. EPG and MF	Clinical protocol (grey zone: 80–95 %)	Research protocol (grey zone: 85–95 %)
Mean eggs counted = 40 • 40 EPG at MF = 1 • 200 EPG at MF = 5 • 400 EPG at MF = 10 • 1000 EPG at MF = 25 • 2000 EPG at MF = 50	9 animals (Total eggs = 360)	14 animals (Total eggs = 560)
Mean eggs counted = 15 • 15 EPG at MF = 1 • 75 EPG at MF = 5 • 150 EPG at MF = 10 • 375 EPG at MF = 25 • 750 EPG at MF = 50	12 animals (Total eggs = 180)	18 animals (Total eggs = 270)
Mean eggs counted = 8 • 8 EPG at MF = 1 • 40 EPG at MF = 5 • 80 EPG at MF = 10 • 200 EPG at MF = 25 • 400 EPG at MF = 50	15 animals (Total eggs = 120)	23 animals (Total eggs = 184)

mean EPG of the test group is known or can be reasonably estimated, and a FEC method is used that has a multiplication factor sufficient to ensure that the required numbers of eggs are counted, then the version with fewer animals will be appropriate. However, we note that predictions of mean FEC before collecting samples will likely be quite inexact, so the mean number of eggs counted per animal indicated here (in the left column of the following tables) should be interpreted as the minimum expected pre-treatment mean, rather than the most likely prediction of the true mean. Similarly, it should be reiterated that regardless of the numbers of eggs counted, testing more animals will almost always improve the likelihood for a conclusive result. Consequently, the group sizes provided in this section should be considered a minimum number; one may always choose to test more animals than the numbers shown here. It should also be understood that the numbers given assume that pre- and post-treatment samples are obtained from all animals: any potential loss to follow up should be compensated for by the inclusion of an appropriate number of additional animals. As discussed previously, we have also set a minimum sample size of five animals for all applications to ensure that analysis programs are able to calculate a reasonable estimate of variability.

The information provided in the boxes for each host animal and/or drug should be interpreted using the following information:

- Target efficacy = the level of FEC reduction observed when the drug was first used and thus what is expected when there is no AR. This value was derived from data reported in published studies at the time of product registration and/or early in the life of the product after registration.
- Lower efficacy target = a value below the 'Target efficacy' that defines the width of the 'grey zone' and therefore impacts the required sample size. The grey zone represents the range of possible true efficacies for which it will be difficult to determine that the observed FECR truly represents either resistance or susceptibility, i.e. efficacies for which we should expect the classification to be *inconclusive*. Increasing the lower efficacy target therefore represents a trade-off between a desire to detect smaller reductions in efficacy (i.e. reducing the grey zone) and increasing logistical demands (increasing group size and number of counted eggs). Differences in this value are the basis of the distinction between the 'clinical protocol' and 'research protocol' provided in these guidelines.
- MF = multiplication factor of the FEC method used to calculate the EPG based on the number of eggs counted.
- Minimum mean eggs counted (provided in the left column) = mean number of eggs counted under the microscope (before applying a correction factor) that is required on average per individual in the group, in order to provide the required total number of eggs counted for the full group.
- Total eggs = the minimum cumulative total number of eggs that need to be counted under the microscope across the tested group.
- Number of animals = the minimum number of animals required for the protocol used (research or clinical), based on the minimum mean eggs counted per animal in that group as indicated in the left column.
  - o Note that as the number of animals tested for a given protocol increases, the total number of eggs required decreases.
- There are several additional points that should be noted:

- o One can reach the minimum number of counted eggs using methods with differing MF depending on the mean pre-treatment FEC (in EPG) of the group.
- o When FEC (in EPG) are low then a lower MF is required, and when FECs are high one can choose a FEC method with a higher MF.
- o The minimum number of counted eggs directly impacts the number of animals that are needed in the test group; as mentioned above, this number is determined by both the pre-treatment mean FEC (in EPG) of the group and the MF of the egg counting method used.
- o Testing more animals, regardless of the pre-treatment mean FEC, will always provide a more precise measurement of FECR; thus testing more animals will lead to a conclusive result more often. Consequently, larger group sizes become increasingly important as numbers of counted eggs decrease.
- o Small group sizes cannot be compensated for by counting many more eggs than are required for a given protocol. In such cases, counting more eggs than is listed in the guideline will provide only a limited improvement in precision.

CRediT authorship contribution statement

Ray M. Kaplan: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Project administration. **Matthew J. Denwood:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Martin K. Nielsen:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Stig M. Thamsborg:** Investigation, Writing – original draft, Writing – review & editing. **Paul R. Torgerson:** Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **John S. Gilleard:** Writing – original draft, Writing – review & editing. **Robert J. Dobson:** Writing – original draft. **Jozef Vercruysse:** Investigation, Writing – review & editing. **Bruno Levecke:** Conceptualization, Methodology, Software, Investigation, Writing – original draft, Writing – review & editing, Project administration.

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 paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109936.

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