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Research paper

World association for the advancement of veterinary parasitology (WAAVP): Third edition of guideline for evaluating the efficacy of equine anthelmintics

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ABSTRACT

This guideline have been developed to assist in the design, execution, and interpretation of studies to assess the efficacy of anthelmintic drugs against internal parasites of equines, including nematodes, cestodes, and larval instars of *Gasterophilus* spp. The design and execution of critical and controlled studies are outlined, and their advantages and disadvantages are discussed. Unique considerations for specific target parasites are included. Information is also provided on selection of animals, procedures for randomization, housing, feeding, dosage titration, dosage confirmation and field studies, record keeping and necropsy procedures. Finally, this document includes guidance for group size determination and statistical analysis of study results. This guideline should assist investigators in the evaluation of anthelmintic drugs in horses by using comparable and standardized procedures in studies with appropriate numbers of animals.

1. Introduction

World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating anthelmintic efficacy in equines were first sanctioned by WAAVP and published in 1988 (Duncan et al., 1988). A second edition followed in 2002 (Duncan et al., 2002). The aim of the current WAAVP guideline is to establish uniform international processes for a meaningful evaluation of anthelmintic drug efficacy, and to incorporate new insights. The general principles of anthelmintic efficacy evaluation and how to conduct studies are outlined in Geurden et al. (2022). Similarly, a WAAVP guideline for conducting fecal egg count reduction test studies are provided in Kaplan et al. (2022), and several of these will apply for the field studies described herein. This document provides detailed guidance relevant to domestic equines and is intended to (1) assist investigators in designing scientifically sound studies to evaluate efficacy of new or existing anthelmintic drugs and (2) provide criteria for determination of test animal group size in terminal studies to ensure appropriate statistical power for the expected magnitude of effect. The guideline presented herein summarize standard methods for anthelmintic efficacy evaluations in equines and outline relevant biological and technical aspects specific to each parasite category.

2. General considerations

The general anthelmintic efficacy guideline includes a protocol checklist in its appendix (Geurden et al., 2022), which is also recommended to be used for the planning of all equine anthelmintic efficacy studies. All animal studies need to be conducted in compliance with existing local and national guidelines governing animal care and use.

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This document considers equines as horses or ponies, but does not cover other equids such as donkeys or mules. In most equine anthelmintic efficacy studies, naturally infected animals are used, but it may be desirable in some cases to evaluate anthelmintic activity against species, such as *Strongylus vulgaris* or *Parascaris* spp. by using induced challenges, as this can help ensure adequacy of infection. In general, studies should aim at documenting treatment efficacy against all parasite species and stages present, when possible.

As described in the general guideline (Geurden et al., 2022), masking of study personnel to the treatment status of individual animals should be achieved by separation of study responsabilities and duties for all types of studies described herein. Any animals that die after treatment during studies should be necropsied to identify the probable cause of death, and all relevant records included in the study file. It is important to ensure correct administration of a given oral anthelmintic by avoiding presence of feed within oral cavities and careful inspection of equines upon administration for compound spillage. Body weights should be accurately determined by use of certified scales, but for field studies, estimation by girth tape can be used as a reasonable estimate of body weight (Górniak et al., 2020).

In general, equines should be observed for several hours after anthelmintic treatment and at least once daily thereafter by trained personnel. All health observations, normal or abnormal, must be documented. All abnormal health conditions observed after initiation of treatment should be documented as Adverse Events, whether or not they are possibly associated with the anthelmintic treatment.

3. Critical and controlled tests

Both critical and control tests have historically been used and either is acceptable for assessing the efficacy of anthelmintics against many important parasites of equines. However, critical tests have found only limited use in recent years. Both test methods have been summarized elsewhere (Drudge and Lyons, 1977). The following sections briefly outline the principles of these two tests and summarize their implications and limitations.

According to its definition (Drudge and Lyons, 1977), the critical test method implies that each equine serves as its own control. Estimation of the anthelmintic activity by the critical test method allows for determination of: 1) spectrum of species of parasites affected, 2) removal efficacy, 3) pattern of discharge of the parasites and 4) physical conditions of the parasites (see Drudge and Lyons, 1977, for details). A description of the methodology for the critical test is included in Appendix 1 (supplementary files).

An obvious advantage of the critical test is that an untreated control group is not needed, which reduces the number of equines required for a study, and it may, therefore, be suitable for small pilot studies evaluating treatment efficacy against luminal stages only. For considerations of group size, refer to section 10.3 of this guideline. An important limitation of the critical test, however, is that it is a time-consuming and laborious method that only allows for evaluation of luminal stages of the parasites. Therefore, it cannot be used to determine anthelmintic efficacy against migrating or encysted worm larvae, which are of particular importance in equine parasite control. The feasibility of critical testing is further limited by the requirement to confine equines individually on limited or no bedding for 6–7 days post-treatment (Drudge and Lyons, 1977), which raises additional issues regarding animal welfare. Furthermore, the critical test requires intensive labor to collect and sieve/wash all feces deposited by each animal, or aliquots thereof. Smaller nematodes normally found in the stomach or small intestine may be partially digested when passed in the feces and can, therefore, be difficult to find, identify, and count accurately. Accuracy and precision of the critical test can also be affected by natural expulsion of parasites during the fecal collection period. For these reasons, the critical test is rarely a preferred choice for equine anthelmintic efficacy studies.

Controlled tests comprise the most widely used experimental design for determining anthelmintic efficacy in equines. For considerations of group size, refer to section 10.3. For considerations of randomizing group assignments, refer to section 4.2. The methodology of controlled tests is detailed in Appendix 1 (supplementary files).

Controlled tests enable determination of anthelmintic efficacy against all parasite species and stages of interest, including migrating and encysted larvae. As larval stages are of significant importance in equines due to their pathogenic potential, the controlled test is the preferred study design for equine anthelmintic efficacy studies.

4. Enrolment of equines in studies

4.1. Selection of animals

Candidate equines should be in good health, with no clinical signs of parasitic disease. Body weights of enrolled equines should be measured prior to treatment for calculation of individual doses. In any study, the animals should have similar parasitological backgrounds, whether the infections are naturally acquired or induced. Although rarely practiced in equines, induced infections can be superimposed on naturally acquired infections to allow testing against as wide a range of parasite species as possible (Reinemeyer et al., 2014). However, the recommendations given in the following all pertain to studies using naturally infected equines.

Efficacy of an anthelmintic may be determined in either horses or ponies. When possible, animals should be obtained from the same source, ensuring exposure to the same parasite community, and be of similar breed/type and age. In general, caution should be exercised when sourcing and relocating animals prior to a study, as stress factors can affect parasite burdens, and significant proportions can be lost prior to initiating the study. For most of the important parasite species, young (1–4 years old), naturally infected animals are likely to have the highest numbers and the widest range of parasite species. However, specific age considerations apply for *Strongyloides westeri* and *Parascaris* spp. (Table 1).

Fecal egg counts may provide evidence of patent infections and can be used to identify suitable candidates for a study, but are not equally

Table 1
Optimal seasons and host ages for studies to evaluate anthelmintic efficacy against various equine gastrointestinal parasites using naturally infected equines. The seasons defined here correspond to temperate climate regions*.

Equines aged one year and older				
Season				
Stage	Spring	Summer	Autumn	Winter
Cyathostomins				
luminal	++	++	++	++
encysted larvae	++	+	+++	+++
Strongylus spp.				
luminal	+++	+	++	+++
migrating larvae	+	+	+++	+++
Cestodes				
luminal	+++	+	++	+++
Gasterophilus spp.				
gastric	+++	–	+	+++
oral	–	–	++	+
Foals/weanlings				
Age (months)				
Stage	0–3	3–6	6–9	9–12
Strongyloides westeri				
luminal	+++	+	–	–
Parascaris spp.				
luminal	–	+++	++	+

- Not recommended.
+ A study is possible, but timing is far from optimal.
++ A study is likely to yield useful data.
+++ Optimal timing for a study targeting this parasite/stage.
* Generally corresponds to both Northern and Southern Hemisphere, although limited data are available from the Southern Hemisphere.

useful for all parasites covered herein.

4.2. Allocation of animals

For controlled tests, animals should be allocated randomly to treated or control groups. Ascarid and strongylid fecal egg counts are poorly correlated to worm burdens (Nielsen et al., 2010), so the use of fecal egg counts for ranking and allocation of equines to treatment groups is only meaningful when egg counts are used as a primary or secondary efficacy endpoint (Geurden et al., 2022). However, it is generally recommended to include determination of fecal egg counts as secondary efficacy endpoints in terminal anthelmintic efficacy studies, which would then justify using fecal egg counts in the group allocation procedure. For studies evaluating parasites in foals (*i.e.*, *S. westeri* and *Parascaris* spp.), it is recommended to block subjects by sex and month of birth and then randomly allocate to treatment or control groups by use of a randomization scheme. For other age groups, equines may be blocked by birth year (or range of years), ranked by decreasing magnitude of body weight or fecal egg count and randomly allocated to treatment or control groups.

4.3. Feeding and maintenance of equines

In general, selection of a ration is at the discretion of the investigator, but the feed should be commonly available, nutritionally adequate for the age and weight of the study animals, and identical for all animals enrolled. The same feed should be used during acclimation and during the testing phase, and qualitative changes are discouraged. Drinking water should be made available on an *ad libitum* basis.

Anthelmintic formulations may be mixed with the animal's normal ration or the anthelmintic active may be incorporated at the manufacturing stage in a proprietary, pelleted medicated feed. A certificate of analysis which reports the active's concentration in the feed should be available, and retention samples from each batch manufactured should be archived for possible feed analysis until a final study report has been issued. Additionally, in studies with a proprietary pelleted feed, the untreated control group should receive a similar diet, which differs only in the absence of the anthelmintic being tested.

If an anthelmintic is administered as a medicated feed, the identical base ration (unmedicated) should be fed during the 7-day acclimation period to allow the animals to become accustomed to the study diet. If a pelleted, top-dress formulation is to be used, all animals should be offered unmedicated pellets during the acclimation/treatment period in the same proportion as the medicated pellets will be offered to the treatment group. The untreated animals will then serve as placebo controls. Whichever method of in-feed administration is used, the anthelmintic should be administered according to manufacturer's instructions, in a small quantity of the ration, before offering the remainder of the unmedicated feed. Animals need to be individually penned during administration of the feed to ensure intake of the entire dose. The time required for complete consumption should be recorded. If the medicated feed is not consumed within a period of time stipulated by the protocol the remaining feed should be removed, and quantities of unconsumed feed should be weighed and recorded.

At the conclusion of a study, feed may be withheld for a defined time-period (*e.g.* 12 h or 24 h) prior to necropsy to reduce the volume of ingesta and, thereby, facilitate parasite recovery and counting. If body weights are recorded post-treatment, these should be measured before withdrawal of feed.

5. Parasite species considerations

The biology of the target parasite(s) must be considered when designing an anthelmintic efficacy study. Various parasite categories may require specific modifications of sampling or processing procedures. Following is a brief synopsis of biological characteristics to consider when planning studies with major equine gastrointestinal parasites.

5.1. Cyathostomins (small strongyles)

Strongylid nematodes infecting equines are traditionally divided into small and large strongyles, based on morphometric factors and migratory behavior. Although the term "small strongyles" is often regarded as a synonym for the subfamily Cyathostominae, this designation may be defined more broadly as non-migratory strongyle parasites, which also includes some members of the Strongylinae subfamily (such as *Triodontophorus* spp.). For studies determining anthelmintic efficacy against cyathostomin nematodes, the norm is to use naturally infected equines. Inducing infections is complicated because cyathostomin-free equines practically do not exist, and the universal presence of encysted and arrested larval stages makes it virtually impossible to eliminate pre-existing infections. Furthermore, all natural infections are comprised of multiple species, making it impossible to determine the appropriateness of the isolates, and field or laboratory isolates are rarely maintained by research groups due to the resources required.

Cyathostomin larval stages deserve specific attention due to their pathogenicity, key role in population dynamics and the practical benefits to be gained from larvicidal efficacy. Foals tend to have fewer encysted larvae overall and a smaller proportion of early third stage larvae (EL3), which may be attributable to a lack of arrested development in the first year of life (Nielsen and Lyons, 2017). Equines 1–4 years of age tend to have high encysted worm burdens and are, thus, most appropriate for larvicidal anthelmintic evaluation (Chapman et al., 2003). Consideration should also be given to seasonality (Table 1), as encysted burdens tend to accumulate over the course of the grazing season in some climates (Ogbourne, 1975; Chapman et al., 2003). Recommendations on the best timing and age of the equines to evaluate efficacy is provided in Table 1. Housing can have a marked impact on encysted cyathostomin dynamics as well. Stall confinement depletes encysted larval numbers over time due to progressive maturation of larval stages. The process by which EL3 develop into late third stage (LL3), and then fourth stage larvae (L4) with subsequent emergence from the mucosa appears to be regulated by complex, yet undescribed mechanisms. The progression of this sequence of events apparently can be accelerated by removal of luminal stages with effective anthelmintic treatment (Eysker et al., 1989), or disrupted if incoming, new larvae are unavailable. Extended confinement, therefore, can falsely increase apparent larvicidal efficacy. Keeping equines at pasture, however, is accompanied by constant ingestion of infective larvae which eventually elevates EL3 counts, and could, therefore, lower efficacy estimates. The time interval from larvicidal treatment to necropsy is critical. A recently conducted larvicidal efficacy study provided meaningful data for both EL3 larvae as well as LL3/L4 larvae with a two-week post-treatment interval between treatment and necropsy. In comparison, a five-week interval resulted in very low larvicidal efficacy estimates (Bellaw et al., 2018). Thus, two weeks is the recommended post-treatment interval for studies intending to estimate larvicidal efficacy (Table 2), and it is recommended that equines enrolled in necropsy studies be confined to stalls or gravel paddocks for the two-week period. Appendix 1 outlines methodologies for enumeration of encysted cyathostomin larvae.

The multitude of cyathostomin species occuring in natural infection constitutes a considerable challenge for anthelmintic efficacy determination, as only for a subset of these species adequate infections will be achieved allowing reliable efficacy determination. A recent meta-analysis of cyathostomin worm count data reported over the last four decades has identified the predominant cyathostomin species on different continents across the world (Bellaw and Nielsen, 2020), as summarized in Table 3. Accordingly, these data indicate which cyathostomin species are the most relevant targets for efficacy evaluation. Based on Table 3, it appears that the three species (occurring with high prevalence and relative abundance *Cylicocyclus nassatus, Cyathostomin*

Table 2
Recommended mean worm counts for adequacy of infection, and post-treatment intervals (weeks) between treatment and necropsy for different categories of equine intestinal parasites. Recommendations are based on yields reported from untreated equines in historic studies[a].

Parasite	Stage	Recommended post treatment interval (weeks)	Recommended minimum mean count[b]
Cyathostomins	Luminal adults	2	10,000[c]
	Luminal L4	2	10,000
	Encysted	2	10,000
Strongylus spp.	Luminal adults	2	50
	Migrating	5	20
Parascaris spp.	Luminal	2	20
	Migrating	5	20
Strongyloides westeri	Luminal	2	50
Habronema spp.	Gastric	2	20
Cestodes	Luminal	2	10
Gasterophilus spp.	Gastric	2	20
	Oral	2	20

[a] Lyons et al., 1974; Drudge et al., 1982; Lyons et al., 1992; Reinemeyer et al., 2015; Bellaw et al., 2018.
[b] Mean parasite counts for the control group (controlled tests) or the total parasites recovered (critical test).
[c] Guideline threshold given for total adult cyathostomin counts. For any given species, the minimum recommended mean worm count is 100.

Table 3
Global prevalence and relative abundance of cyathostomin species reported in necropsy studies during 1975-2020 (Bellaw and Nielsen, 2020). Species not listed here were in the Very Low category with prevalences <20 % and relative abundances <0.3 % and are unlikely to be prevalent and abundant enough to allow an estimation of anthelmintic efficacy.

Level*	Species	Prevalence (%)	Relative abundance (%)
High	*Cylicocyclus nassatus*	93.4	20.3
	Cylicostephanus longibursatus	92.9	19.2
	Cyathostomum catinatum	90.6	16.4
	Cylicostephanus goldi	81.6	6.0
Moderate	*Coronocyclus coronatus*	76.7	3.8
	Cylicostephanus calicatus	76.5	3.4
	Cylicostephanus minutus	71.4	4.4
	Cylicocyclus leptostomum	62.6	4.0
	Cylicocyclus insigne	36.0	2.1
	Coronocyclus labratus	31.0	0.7
Low	*Cyathostomum pateratum*	30.1	1.3
	Coronocyclus labiatus	28.7	1.2
	Cylicocyclus ashworthi	23.6	0.7

* Thresholds for the three categories are as follows: Prevalence: high = > 85 %, moderate = 40–85 %, low < 40 %. Relative abundance: high = >12 %, moderate = 4–12 %, low < 4%.

catinatum, and *Cylicostephanus goldi*) are likely study targets, while meaningful data could be generated for the five species in the moderate category. In contrast, it may not be feasible to provide efficacy data for the remaining 32 cyathostomin species (Table 3). Identification of adult cyathostomins to species requires a high level of specialized expertise, which often challenges the feasibility of such studies. Molecular techniques such as the reverse line blot assay (Cwiklinski et al., 2012) and next-generation meta-barcoding (Poissant et al., 2021) can be used to identify and semi-quantify the cyathostomin species present and may find use in the future.

It should be emphasized that luminal L4s warrant specific attention due to putative, decreased efficacy of some anthelmintics (Lyons et al., 2009; Bellaw et al., 2018), so it is important to generate and report efficacy data for this stage for new anthelmintic drugs as well.

Historically, there has been some interest in the assessment of persistent efficacy for anthelmintics against cyathostomin infections. However, given the complexity of cyathostomin biology outlined in this section, and especially the species diversity and the variable factor of arrested development, the authors of this document are not able to propose a meaningful study design for determination of persistent efficacy against cyathostomin infections. The strongylid egg reappearance period (ERP) should be determined for any equine anthelmintic product (see Section 8) and will reflect a combination of larvicidal efficacy, arrested development of EL3s, and persistent efficacy of the anthelmintic drug.

5.2. Large strongyles

Although large strongyles of the subfamily Strongylinae comprise 14 species (Lichtenfels et al., 2008), this term is often applied as a synonym for the three migratory species of the genus *Strongylus* only: *S. vulgaris*, *S. edentatus*, and *S. equinus*. Although the *Strongylus* genus is generally considered rare in managed equine populations today, *S. equinus* is encountered extremely rarely, while *S. vulgaris* and *S. edentatus* can still occur in equines receiving little or no anthelmintic treatment. *Strongylus vulgaris* often receives primary attention due to its pathogenicity and is traditionally included in anthelmintic efficacy evaluations. Induced infections with *Strongylus* spp. have been described (Duncan and Pirie, 1972; Slocombe and McCraw, 1984), but the long prepatent periods can require up to a year before adequate adult infections have been established. With natural *S. vulgaris* infection, some seasonality is observed, with relatively more migrating larvae occurring over winter, and a higher proportion of adults during summer (Duncan, 1974), and this should be considered when scheduling a study aiming at generating efficacy data for the different stages of this species (Table 1).

If larvicidal efficacy against *S. vulgaris* is to be evaluated, the time interval between anthelmintic treatment and necropsy requires consideration (Table 2). Early studies with ivermectin, for example, suggested no larvicidal effectiveness against L4s at the two-week post-treatment interval, but high efficacy was demonstrated, when equines were necropsied five weeks post-treatment (Slocombe and McCraw, 1984). Furthermore, if induced infection with *S. vulgaris* is used, at least three months must be allowed for normal larval migration before evaluating efficacy against the migratory L5 stage. Historically, larvicidal efficacy of macrocyclic lactones was only evaluated against *S. vulgaris* L4s (Slocombe and McCraw, 1984; Klei et al., 1984), but a recent study suggested that ivermectin has little or no efficacy against migratory L5s (Nielsen et al., 2015). Thus, it appears that efficacy against the L4 stage cannot be extrapolated to the migratory L5, and that larvicidal efficacy, therefore, should be evaluated against all migratory stages, if possible. Methodology for recovering, identifying, and enumerating *Strongylus* spp. stages is described in Appendix 1.

5.3. Parascaris spp

Equine ascarids are major parasitic pathogens and are often target parasites in anthelmintic efficacy studies. The two equine ascarid species, *Parascaris equorum* and *P. univalens* can only be differentiated by karyotyping (Goday and Pimpinelli, 1984; Nielsen et al., 2014). This procedure requires viable primordial germ cells or eggs (Goday and Pimpinelli, 1984), and has not been part of any efficacy study in the past. Without species determination by karyotyping, a study can only allow conclusions at the *Parascaris* genus level. Recent findings suggest that *P. univalens* is the predominant species across the world, and that *P. equorum* is only rarely encountered, if ever (Nielsen et al., 2014; Martin et al., 2018).

Age is an important confounder with regard to equine ascarids because most natural infections are eliminated due to an age-dependent immunity by about 6–8 months of age (Fabiani et al., 2016). The

preferred age range for naturally infected foals to be enrolled in anthelmintic efficacy studies is, therefore, 3–6 months (see also Table 1). Foals older than this age range can be enrolled if they are shedding ascarid eggs, but it is imperative that animals in the control group are always age-matched with treated animals. Furthermore, female foals have been reported to harbor larger ascarid burdens than males (Fabiani et al., 2016), so it may be recommended to block groups by sex as part of the group allocation procedure.

Induced inoculation can be used to ensure more uniform exposure to ascarids (DiPietro et al., 1988) and may also expand the age range of potential enrollees. However, it is recommended that equines less than 12 months of age be used for induced infections, as age-dependent immunity is usually established in equines older than one year (Clayton and Duncan, 1979).

Methods for evaluation of larvicidal efficacy against *Parascaris* migrating larval stages in the liver and lungs have been described (Lyons et al., 1976), but several studies reported low or no larval yields with this approach (DiPietro et al., 1987, 1988; Vandermyde et al., 1987). Accordingly, these methods are considered unsuitable and are not recommended for anthelmintic efficacy evaluation. A more appropriate and recommended study design for evaluating lefficacy against migrating larval stages is to necropsy the equines at 4–5 weeks post-inoculation with recovery of luminal *Parascaris* spp. L4s from the small intestine (Lyons et al., 1976; DiPietro et al., 1987). For evaluation of efficacy against luminal larval stages, however, the recommended interval between treatment and necropsy is two weeks (Table 2).

5.4. Stomach worms

Equine stomach worms include the Spiruridae family nematodes *Habronema* spp., and *Draschia* spp. as well as the trichostrongylid nematode *Trichostrongylus axei*. Traditionally, anthelmintic efficacy against these parasites has been evaluated in naturally infected equines. The presence of *T. axei* can be confirmed by coproculture, but validated *in vivo* diagnostic techniques do not exist for the spirurid nematodes. Mucosal digestion (Drudge et al., 1963) and saline incubation (Rehbein et al., 2013) techniques have been described for recovery of *Habronema* spp., *Draschia* spp., and *T. axei* specimens at necropsy. See Appendix 1 for further detail.

5.5. Strongyloides westeri

Induced infections with *S. westeri* have been reported (Lyons et al., 1973), and, although not widely implemented, this approach has been utilized in some anthelmintic efficacy studies (Drudge et al., 1982). In natural infections, this parasite is foundprimarily in very young foals (Lyons and Tolliver, 2014). Thus, suckling foals less than three months old would be the ideal subjects (Table 2). See Appendix 1 for the methodology used for recovery and enumeration of *S. westeri* from the small intestine.

5.6. Oxyuris equi

Pinworms tend to be sporadic in occurrence (Bucknell et al., 1995; Lyons et al., 2007), and induced infection of equines with *O. equi* has not been established as a useful method for anthelmintic efficacy studies. Natural infection is the only option, but, in the absence of a validated *in vivo* diagnostic technique, it is a challenge to enroll animals with confirmed infections. In general, five months are required for maturation of *O. equi*, and there is a short period of development within the intestinal mucosa before fourth stage larvae emerge into the intestinal lumen. Adult pinworms are most commonly found in older foals and yearlings, whereas large numbers of larvae (without mature worms) are often present in older animals (Duncan et al., 2002).

5.7. Cestodes

Although several tapeworm species can occasionally infect equines, this section pertains to cestode species classified in the family Anoplocephalidae. Of the three anoplocephalid species infecting equines, *Anoplocephala perfoliata* is by far the most common and abundant. Of the other two species, *A. magna* is sporadically reported in some countries, predominantly among horses less than two years of age (Meana et al., 2005). *Anoplocephaloides mamillana*, on the other hand, is very rare and sporadic in occurrence. Further considerations for recovery and enumeration of *A. perfoliata* are included in Appendix 1.

Infections with anoplocephalid tapeworms are acquired during grazing by ingestion of forage contaminated with oribatid mites containing the cysticercoid larval stage. Horses older than 6 months of age may be infected with adult worms. After 1–2 months after ingestion of infected oribatids, mature tapeworms may be found in the small intestine and cecum and segments and/or eggs are passed in the feces (Duncan et al., 2002). A standardized model for induced inoculation has yet to be established. Accordingly, natural infection is the only viable experimental option.

Seasonality should be considered when planning a tapeworm study, and cestode populations are renewed on a yearly basis (Meana et al., 2005). Thus, tapeworm burdens are likely to be the highest in late autumn, winter and spring, and lowest during summer and early autumn (Nilsson et al., 1995; Meana et al., 2005; Tomczuk et al., 2015). Accordingly, studies should not be conducted during times of expected transition between parasite generations, *i.e.* late spring/early summer (Table 1).

5.8. Gasterophilus spp

Parasitic larvae of botflies of the genus *Gasterophilus* commonly occur in equines and are often considered in anthelmintic efficacy studies if the compounds tested have insecticidal activity. Female *Gasterophilus* flies lay eggs on the hairs of the horse during the summer months; upon ingestion, hatched larvae migrate from the mouth to the stomach where they spend approximately 10 months before being passed in the feces (Duncan et al., 2002). Induced infections have been used in studies evaluating efficacy against *Gasterophilus* spp. (Drudge et al., 1972, 1975), but have not been employed in recent decades.

Of the various species infecting horses, only two (*Gasterophilus intestinalis* and *G. nasalis*) are likely to be sufficiently prevalent and numerous to allow for demonstration of significant treatment effects. Other species, such as *G. pecorum* or *G. hemorrhoidalis*, can still be found in some geographic locations and can potentially be included in studies conducted therein. Botfly larvae exhibit a distinct seasonal pattern in which infections are acquired in the late summer and early autumn. After migration and molting in the oral cavity for a few weeks, the larvae of *G. intestinalis* and *G. nasalis* are established in the stomach and pyloric region, respectively, by mid to late autumn and remain there until the following spring. Consequently, few or no L2 and L3 larvae are present during summer and early autumn, so the recommendation is to conduct anthelmintic efficacy studies during winter or early spring months (Table 1).

The oral early instars stages of *Gasterophilus* can have pathogenic potential (Cogley, 1989; Vemming et al., 2015) and therefore it may be desirable to conduct efficacy studies against oral stages. Tongues and interdental spaces can be inspected for the presence of larvae (Cogley, 1989), but such studies would need to target a very specific time frame in the late summer and early autumn, when early instars are present. An established alternative to recovering larval stages from the oral cavity is to allow sufficient time (three weeks) following treatment for the larvae to progress to the stomach, from where they are more easily recovered and enumerated (Drudge et al., 1972).

5.9. Other parasites

The equine lungworm, *Dictyocaulus arnfieldi*, is only rarely included in anthelmintic efficacy studies, as this parasite rarely completes development to the adult stage in horses. However, anthelmintic efficacy may be determined in donkeys (Clayton and Neave, 1979), which are considered the natural host for this parasite, and a few studies have been done in horses as well (Lyons et al., 1985; Britt and Preston, 1985).

The liver fluke, *Fasciola hepatica*, is recognized to be of clinical significance in horses in some regions (Howell et al., 2020), but so far, no reliable methodology for diagnosis and enumeration can be recommended.

6. Dosage determination studies

Dosage determination (DD) studies (sometimes referred to as dosage titration trials) are usually conducted as controlled tests, and typically follow pilot studies which have demonstrated preliminary activity of the anthelmintic and identified an approximate dosage. Ideally, dosage determination studies should evaluate efficacy against all target parasites. If broad-spectrum activity of the anthelmintic is anticipated, a secondary objective can be to define the dose-limiting parasite. This is defined as the specific targeted parasite or parasite stage (larval or adult) requiring the highest effective dose (Geurden et al., 2022). As outlined in the previous section, the target spectrum may be incomplete because naturally infected horses are most often used.

Studies should be carried out with a range of dosages bracketed around an effective dosage identified by pilot studies. Animal group sizes are based on the considerations for adequacy of infection presented in section 10.4 regarding the parasitic stages, species or groups of interest.

A DD study should include a minimum of three groups receiving different dosages as well as an untreated control group. One group should be treated with the anticipated recommended dosage of the final formulation, while those of the second and third groups should be treated with a lower and a higher dosage (Geurden et al., 2022). The route of administration and the formulation of the product should be those proposed for marketing. The efficacies of each dosage should be determined as outlined in section 3.

7. Dosage confirmation studies

Dosage confirmation (DC) studies should employ the final or near-final product formulation, administered at the proposed label dosage and *via* the indicated route of administration. Studies with naturally infected animals should source the study animals at different locations using indigenous or geographically unique field isolates of the target parasites.

In both DD and DC studies, the required group size for determining anthelmintic efficacy should be chosen following the guidance presented in section 10.3. The selected dosage of the anthelmintic is administered to each animal in accordance with the provided label instructions.

An efficacy claim against immature stages should refer to the stage of development (in the case of natural infections) or to the age of the parasite in days (in the case of induced infections) at the time of treatment.

Although claims for efficacy against the major equine parasites should be genus, species and/or stage specific; it may be acceptable to include data for several species of cyathostomins and non-migratory Strongylinaeas one group ("small strongyles"), because their biology and location within the host gastrointestinal tract are similar. Specific considerations for cyathostomin species are presented in section 5.1 and Table 3.

8. Field studies

Field studies are conducted to further evaluate the performance of a product as used in the field, and to evaluate its safety when used in different breeds, types, and ages of equines. Field studies should be designed and conducted with various breeds or types of animals in different geographical locations to account for differences in environmental conditions, feeding and management practices, parasite species diversity of parasite populations, and different anthelmintic resistance profiles. Animals in the treated group should receive the recommended dosage of the final formulation or authorized anthelmintic administered in compliance with (proposed) label instructions. Management of all animals enrolled in a clinical study should be similar within each participating site.

In field studies, anthelmintic efficacy is usually determined by fecal egg count reduction (FECR), which in equines is limited to ascarid and strongyle type egg counts. For these studies, it is generally recommended to follow the recently published WAAVP guidelines for Fecal Egg Count Reduction Test (FECRT) studies (Kaplan et al., 2022). This includes consideration of the number of parasite eggs counted (raw counts prior to applying the multiplication factor) within the experimental group prior to administration of the test compound and provides guidance for appropriate statistical analysis with links to useful online interfaces for this purpose. However, some aspects may differ for the field studies considered here. First of all, the FECRT guidelines recommend group sizes based on the expected efficacy level for each anthelmintic drug class against each parasite category (Kaplan et al., 2022). A field study aims to establish field efficacy for a given anthelmintic, whereas the aim with an FECRT study is to detect a reduction from an established baseline efficacy. Thus, while the methods for calculating FECR means and associated confidence (or credible) intervals should be identical, the group size considerations may differ between the two types of studies because of the different aims (see Section 10.3 for further details). For determination of strongyle FECR, an untreated control group is not necessary, and anthelmintic efficacy is, instead, evaluated based on pre-and post treatment FEC of treated animals. However, for determination of ascarid FECR, age matched controls are required at a ratio of at least one control for every three treated animals. For ERP determination studies, it is also recommended to include untreated control animals (see below).

At a minimum, fecal egg counts of each animal should be determined at the time of dosing (Day 0) and again 14 days after treatment (Day 14). Meta-barcoding techniques for identification of strongylid species (Poissant et al., 2020) are encouraged as a means of characterizing the parasite population studied both pre-treatment and during monitoring for egg reappearance.

It is recommended that the ERP is established for new equine anthelmintics. Fecal egg counts should be monitored at weekly or biweekly intervals for up to 3–4 months post-treatment, depending on the expected persistent activity of the anthelmintic product under evaluation. A definition for ERP is when the mean percentage of FECR for the treated group falls below a threshold set 10 % below the FECR determined at two weeks post-treatment (Nielsen and Reinemeyer, 2019). Thus, ERP should be reported as the number of weeks post-treatment, for which the upper confidence (or credible) interval (CI) for the mean FECR falls below the mean FECR determined at 14-day post-treatment minus 10 %. For example, if the mean FECR for a given anthelmintic is determined to be 99.5 % at 14 days post-treatment, the threshold for the upper CI is 89.5 %. See section 10.2 for further details on the statistical analysis.

It should be noted that cyathostomin prepatent periods and ERPs may depend largely on the age of the equines studied. In a group of 4–5-year old ponies, Smith (1976) determined that strongylid egg shedding resumed at 12–15 weeks post-inoculation. However, when he repeated the study protocol with the same ponies six years later, the prepatent period/ERP had increased to 17–18 weeks (Smith, 1978). Thus, ERP data should always include the age range of the equines studied. With

ascarids, ERP is not a meaningful measure because of the potential influence of host immune response.

The principle of the new WAAVP guidelines for FECRT studies is to not recommend any specific techniques for fecal egg counting, but rather focus on the number of eggs counted prior to conversion to eggs per gram of feces, and leave it up to investigators to choose a technique most likely to yield the desired number (Kaplan et al., 2022). While this approach focuses the attention on the detection limit (and, hence, multiplication factor) of these techniques, investigators are encouraged to consider other diagnostic performance parameters as well. In general, use of egg counting techniques for which accuracy and precision have been determined and validated in equines is recommended. See Noel et al. (2017) for examples of how these can be determined. As a general guidance, techniques performing with a coefficient of variation (CV) of 20 % or less are considered high precision techniques and would be recommended for this type of study. Accuracy of the egg counting technique is less important than precision but has shown to differ substantially between techniques (Bosco et al., 2018), and it is recommended to select a technique performing with an accuracy exceeding 50 %. It is recommended to use the same fecal egg counting technique at all study sites and use these throughout the studies. The choice of technique should be clearly documented and reported.

9. Records and reports

Complete records should be maintained of all experimental procedures used in an anthelmintic efficacy study. These records include: (1) number, source, breed, age, weight and sex of experimental animals, (2) inclusion and exclusion criteria for enrollment, (3) housing, feed/water and management conditions, (4) number and history of infective inocula if induced infections are used, (4) lot number, expiry date, storage conditions, formulation types, active ingredient(s) and concentrations of the products tested, target dosage and route of administration, (5) adverse reactions if they occur, (6) diagnostic techniques, necropsy procedures, gross pathological changes, identification and counts of parasites recovered by total or aliquot counts; (7) any other information, such as dates, duration and site of the study, responsible personnel, ethical approval documentation, statistical analysis, disposal of carcasses and daily health records. A study report, signed by the clinical investigator and responsible sponsor personnel, should be issued after all components of a study have been completed according to the protocol.

10. Statistical analyses

Fundamentally, the efficacy estimates (*i.e.*, percent reduction of parasite counts of treated animals compared to parasite counts of untreated controls) derived from studies having a control group should consider a central tendency and variation estimate. The decision on which inferential model to be used should incorporate the type of data distribution.

10.1. Central tendency estimates

The conventional method for calculating the group mean parasite (worm) count has been using geometric means (Smothers et al., 1999). The main reasons for using the geometric mean over the arithmetic mean are that the calculation is less affected by outliers and that parasite count data typically have a non-Gaussian and over-dispersed distribution (Sréter et al., 1994; Galvani, 2003). Combined with the limited number of animals per group, using the arithmetic mean or median as a central tendency estimate may underestimate true values of anthelmintic efficacy. However, modern statistical approaches can effectively address this issue by modelling the actual distribution of counts (Alexander, 2012; Love et al., 2017), allowing for an appropriate evaluation of efficacy by using model-generated means (see section 10.2).

10.2. Statistical analysis

For both controlled and critical test studies, it is recommended that differences in parasite counts be analyzed between groups (for controlled tests) or between excreted and total parasite counts (for critical tests). Since parasite count data are rarely normally distributed, several possible approaches are feasible: 1) non-parametric tests, 2) parametric tests following log-transformations of parasite counts to approximate a normal distribution, or 3) using statistical models more appropriate with the specific data distribution, such as negative binomial distribution. While all three options can be justified, recent work with analyzing FECRT data have demonstrated the feasibility of option 3 (Denwood et al., 2010; Love et al., 2017) and similar approaches could, therefore, be taken for analysis of worm count data. Investigators are encouraged to work with statisticians to identify the most appropriate method of statistical analysis for their data.

For calculation of mean FECR, the WAAVP (Kaplan et al., 2022) recommends the use of statistical packages taking into account between-animal variation and Poisson error associated with fecal egg counting methods, such as the hierarchical Bayesian "eggCounts" package (Torgerson et al., 2014; Wang et al., 2018). This package generates model-estimated means with corresponding credible intervals, which are useful measures of FECR precision. As outlined in section 8, it is recommended to use the same statistical method for ERP studies as well.

10.3. Estimation of required group size

The deciding factor for the required group size is the standard deviation (SD) relative to the mean count for each group. In equine parasitology worm counts, it is not unusual for the SD to exceed the mean by 100 % or more, and scenarios with the SD being smaller than the mean are exceptions, (Xiao et al., 1994; Monahan et al., 1996; Reinemeyer et al., 2015). Table 4 provides examples of group sizes required for achieving an acceptable statistical power for given ranges of SD. When the SD is less than the mean count (*i.e.*, relative SD less than 100 %), the required group sizes are much more realistic and practical. However, with the natural infections most often used in equine studies, this is not always the case.

For field studies, the target efficacy endpoint is the FECR. The WAAVP FECRT guideline paper presents suggested group sizes for evaluation of anthelmintics with expected FECR levels ranging from 98 % to 99.9 %, and recommend group sizes of 10–20 equines for most scenarios (Kaplan et al., 2022). However, the aim of FECRT studies to evaluate the presence of AR differs from that of a field studies to confirm

Table 4
Examples of group sizes required for determining a statistically significant reduction in worm counts between treatment and control group means with the probability of type I error set to $\alpha = 0.05$ and the probability of type II error set to $\beta = 0.80$.

			Observed efficacy [3]			
Mean [1]	Standard deviation [1]	CV[2]	99%	95 %	90 %	85 %
	50,000	50 %	3	4	6	8
	75,000	75%	3	4	6	9
	100,000	100 %	3	5	8	11
100,000	125,000	125%	4	7	11	15
	150,000	150 %	5	9	14	21
	175,000	175%	5	10	16	23
	200,000	200 %	6	12	18	25
	CV treated group:		62 %	153 %	150 %	149 %

[1] Examples were chosen to represent a typical mean for cyathostomin worm counts and standard deviations ranging from 50 % to 200 % of the mean.
[2] Coefficient of variation.
[3] Observed efficacies were selected to represent efficacy percentages typically observed in anthelmintic efficacy studies.

anthelmintic efficacy. Larger group sizes can be considered for field studies, as this will increase the precision of the FECR estimate. The ultimate choice of group size should depend on 1) expected FECR, 2) a number of eggs counted (and hence, fecal egg counting technique chosen), and 3) desired precision of FECR estimate (as expressed by width of the confidence (or credible) intervals). Similarly, for studies aiming to determine strongyle ERP, the group size and number of eggs counted are the two factors affecting the precision of the weekly post-treatment FECR estimates. For ERP studies, a minimum of 20 equines per group is generally recommended, but the ultimate decision should be based on the factors outlined above. Given the longer study duration required for ERP determination, it is recommended to keep an untreated age- and FEC-matched control group, if possible, as seasonal fluctuations in strongyle egg shedding may occur.

The importance of inferential statistics permits the observed results to be considered "real" (*i.e.*, less than a 5% chance of being incorrect using an alpha of 0.05) *versus* occurring by chance alone. Statistical analysis for differences between groups should always be completed and results included in the report. If an observed reduction did not reach statistical significance, this should be disclosed.

10.4. Adequacy of infection

When the occurrence of any given target parasite species or stage is less than 100 % among experimental subjects, the group size and statistical power are effectively reduced. If 100 % occurrece of the target parasite species/stages is not achieved in the study subjects, investigators need to clearly disclose this.

In a manner similar to the eggs counted approach now taken for FECRT studies (Kaplan et al., 2022), similar considerations should be given to the number of worms counted. If the number is low, a relatively high SD can complicate a determination of treatment efficacy with statistical significance. This is particularly relevant in equines, as many target species or stages may not be very abundant in naturally infected animals. Therefore, Table 2 provides guidance on the recommended minimum numbers of parasites to be counted. As outlined above, a low mean count can still be useful for a study, as long as the SD is correspondingly low, and preferably lower than the mean. However, counts close to the detection limit of a given technique tend to have relatively larger SDs, so minimum threshold counts are given here. In general, cyathostomin counts (both luminal and encysted larval counts) are estimated by analyzing a subsample (see Appendix 1 in supplementary file) and subsequently applying a multiplication factor to achieve a total count for the given organ or equine. In comparison, larger parasites such as tapeworms, bots, adult *O. equi*, and *Strongylus* spp. are estimated by searching through the entire gastrointestinal content and inspection of mucosal barriers for attached parasites, so no adjustment or multiplication factor is needed (Appendix 1).

11. Conclusions

In these revised guidelines, considerations are provided relevant to equine parasites. Design and execution of equine anthelmintic efficacy studies are challenged by several factors, including the multitude of cyathostomin species, the lack of useful induced infection protocols, and the variation in occurrence and abundance of target parasites in study populations. Furthermore, the current array of ante-mortem diagnostics is limited and does not provide all the desired information about a given parasite species and/or stages within a study population. This diminishes investigators' confidence of ensuring adequate infections with all target parasites in a study. Finally, key anthelmintic product performance features relevant to equines, such as larvicidal efficacy and ERP, require additional procedures or even separate studies.

These guidelines provide investigators with a scientific framework to achieve the best possible quality data in studies for evaluating equine anthelmintic product efficacy. Compared to previous guideline documents, the information on relevant aspects of parasite biology and host/parasite interaction patterns has been expanded, with emphasis on the statistical power achieved in the study.

Data availability

Data will be made available on request.
The data that has been used is confidential.

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.

CRediT authorship contribution statement

Martin K. Nielsen: Writing - original draft, Writing - review & editing. **Georg von Samson-Himmelstjerna:** Writing - review & editing. **Tetiana A. Kuzmina:** Writing - review & editing. **Deborah C.K. van Doorn:** Writing - review & editing. **Aranzazu Meana:** Writing - review & editing. **Steffen Rehbein:** Writing - review & editing. **Timothy Elliott:** Writing - review & editing. **Craig R. Reinemeyer:** Writing - review & editing.

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Appendix A. Supplementary data

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