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World Association for the Advancement of Veterinary Parasitology (WAAVP): Second edition of guidelines for evaluating the efficacy of anthelmintics for dogs and cats

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ABSTRACT

These revised guidelines have been developed to assist in the design, execution, and interpretation of studies to assess the efficacy of anthelmintic drugs against internal parasites in dogs and cats. The design and execution of studies are outlined and discussed. Considerations for specific targeted parasites are included. Information is provided on the principles of selection of animals, procedures for randomization, housing, feeding, necropsy procedures, and record keeping for dose determination dose confirmation studies and field studies. Complementary to the WAAVP general anthelmintic guidelines, these species-specific guidelines should assist investigators in the evaluation of anthelmintic drugs in dogs and cats by using comparable and standardized procedures in studies with appropriate numbers of animals.

1. Introduction

The World Association for the Advancement of Veterinary Parasitology (WAAVP) published guidelines for testing anthelmintic efficacy in dogs and cats (Jacobs et al., 1994), as well as other species including ruminants (Powers et al., 1982; revised by Wood et al., 1995), swine (Düwel et al., 1986; revised by Hennessy et al., 2006), horses (Duncan et al., 1988; revised by Duncan et al., 2002; revised by Nielsen et al., 2022), and poultry (Yazwinski et al., 2003). The WAAVP anthelmintic guidelines aid investigators conducting research in establishing homogeneous approaches for the efficacy assessment of either single compound or combination anthelmintic products, and to minimize sources of variability within and between studies to facilitate comparisons (Vercruysse et al., 2001; Vercruysse et al., 2002). For combination anthelmintic products, additional recommendations are provided in the relevant WAAVP guidelines (Geary et al., 2012).

The present dog and cat species specific guidelines need to be regarded in parallel to the general guidance (Geurden et al., 2022). The current review of the WAAVP anthelmintic guidelines include an evaluation of scientific and technological advancements with updates based on an evaluation of the benefits of the proposed changes, the historical context, the levels of validation, and animal welfare considerations. Statistical considerations are included in the current guidelines because they are essential to the development of protocols, the analysis of data, the determination of efficacy, and, in some cases, the determination of adequacy of infection.

These species-specific guidelines review and discuss the modalities for anthelmintic efficacy assessment in dogs and cats. They concern not only gastro-intestinal helminthoses, but also other helminthic infections such as lungworm infections and dirofilariosis.

2. General principles regarding anthelmintic efficacy studies

The general principles of anthelmintic efficacy evaluation are discussed in more detail in the WAAVP general anthelmintic guidelines (Geurden et al., 2022). In short, the following key principles must be

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adhered to:

- All anthelmintic efficacy studies should be conducted in accordance with local regulations regarding animal welfare and experimentation on animals. The principles of good clinical practice (GCP) (as detailed in VICH Guidelines 9) should be followed for all field studies and may be applied to experimental (syn. laboratory) efficacy studies.
- The study protocol should be finalized before initiating the study. The protocol defines the study objective(s), including the parasite species and parasite stage(s) against which the drug will be evaluated. Information on the number, breed, age, and sex of animals to be used, as well as main husbandry procedures should be provided. The protocol also should define whether induced (syn. artificial, experimental) or natural infection will be used, and provide the methods to infect and/or confirm infection in study animals, as well as the methods to recover and enumerate the target parasites. The study protocol should clearly describe the experimental design, including information on the experimental unit, the test product, type of control group, methods of randomization and allocation to treatment groups, and efficacy calculation. The analysis of the data (e.g., statistical analysis of parasite stages or egg/larval counts) should be clearly defined. Animals should be pair or group housed whenever possible, nevertheless single housing should be considered when comingling may introduce a bias, e.g., to minimize inter-individual test product contamination, or to allow individual health observation and sample collection. Relevant information (e.g., timing, method, and level of accuracy of individual weighing) regarding dose calculation and test product application should be provided.
- Safety observations should be planned in consideration of the known characteristics of the test product (e.g., pharmacokinetics (PK) and tolerance to the components), and the administration site should be identified and examined for possible reactions.
- A study report needs to be written and finalized at the end of the study. It includes an evaluation of whether the study objectives as defined in the protocol were met, provides all relevant details on the study conduct, and provides sufficient detail to allow an independent evaluation of the study design, conduct, results, and conclusions. The study report should also document information about the test product including the trade name (if applicable) and the chemical name of the active ingredient(s), manufacturer, lot number and expiry/retest date.
- For all studies, experimental or field studies, ethics approval must be obtained from the investigator body (e.g. University, Private Contract Research Organization) and the sponsor if any (e.g. pharmaceutical industry, research organization). Ethics should confirm the necessity of animal use, the human care of the animals, and approve the number of animals and the design and methodology of the study. All possibilities to limit the use of animals should be considered.

3. Experimental studies with induced or natural infections (dose determination and dose confirmation studies)

3.1. General principles for dose determination and dose confirmation studies

The primary objective of a dosage determination study is to define the minimum dose of an active ingredient necessary to achieve the desired efficacy against the target parasite(s) (Geurden et al., 2022). If a broad-spectrum activity is targeted for an active ingredient, the least sensitive or dose-limiting parasite of the target spectrum as determined from previous data should be used (more than one parasite may be dose-limiting parasite for an active ingredient).

To provide independent substantiation of efficacy results, at least two supportive dose confirmation studies following similar protocols per parasite species and/or developmental stage for which efficacy is evaluated are recommended, using the final formulation at the minimum recommended dose, dose regimen and route of administration. To demonstrate the efficacy against a targeted parasite species, it is recommended to conduct the two dose confirmation studies with different isolates of relevant geographical regions (i.e. originated from different parts of the world: Europe, North America, Central and South America, Africa, South-East Asia, China and Central Asia, Australia – Pacific, Japan). When a non-terminal natural infection model is possible, it should be used in at least one of the two dose confirmation studies. For parasite species with a very low prevalence or a limited geographical distribution (i.e. “rare” parasite species), a single dose confirmation study may be acceptable instead of two studies. A dose determination study may serve as a dose confirmation study if performed within adequate quality and regulatory premises (e.g., using the final formulation).(Table 1,2).

In experimental efficacy studies, animals can either be infected experimentally or be naturally infected. In studies with natural infections, the individual animal susceptibility and variability observed in the field is reflected. As defined in the general guidance (Geurden et al., 2022), in studies using induced (artificial, experimental) infections, recent field isolates (<10 years since isolation from the field, <5 years for *Dirofilaria immitis*) are generally recommended. The use of laboratory isolates (> 10 years since isolation from the field) may be justified based on the specific study objective (e.g., efficacy against resistant isolates) or if a recent field isolate cannot be sourced. The details regarding the isolate source (year and location of initial isolation), previous drug exposure (if known), resistance status (if known) and maintenance after isolation should be recorded.

Because precise diagnosis of larval stage (pre-patent) infection in naturally infected animals is impracticable, studies with induced infections are generally recommended for efficacy testing against larval stages. The treatments need to be administered at least before the end of the pre-patent period, which can be confirmed by a negative diagnostic test for shedding stages (e.g. eggs, larvae). To target different larval stages, the timing between infection and treatment is based on the

Table 1
Recommended time of treatment in dogs after infection to assess efficacy against adult or larval helminth stages.

Parasite	Adult stages	Larval stages
Gastrointestinal tract		
Strongyloides stercoralis	5–9 days	
Aancylostoma caninum	> 21 days	6–8 days (L4). For somatic larvae, treat within 2 days prior to parturition
Ancylostoma braziliense	> 21 days	
Uncinaria stenocephala	> 21 days	6–8 days (L4)
Toxocara canis	49. days	6–8 days (L4) 3–5 days (L3/L4) 14–21 days (L4/L5) 20–40, usually 30 days (L4)
Toxascaris leonina	70 days	35 days (L4)
Trichuris vulpis	84. days	
Echinococcus granulosus	28 days	
Taenia spp.	> 35 days	
Circulatory system		
*Dirofilaria immitis**	180–210 days	70–120 days (L5), 220 days (microfilariae)

* A range of 30–100 L3 applied sub-cutaneously per dog for preventive studies. A range of 30–50 L3 per dog may be used for adulticide studies but such infections are less useful. If infection by intravenous transplantation of 7–10 pairs of adult worms is used, the worms should be at least 210 days old and an additional 42 days is needed for the dogs to have at least 300 mf/mL before treatment is started.

Table 2
Recommended time of treatment in cats after infection to assess efficacy against adult or larval helminth stages.

Parasite	Adult stages	Larval stages
Gastrointestinal tract		
Ancylostoma tubaeforme	> 21 days	6–8 days (L4)
Ancylostoma braziliense; A. ceylanicum	> 21 days	6–8 days (L4)
Toxocara cati	60 days	3–5 days (L3/L4) 28 days (L4/L5)
Toxascaris leonina	70 days	35 days (L4)
Strongyloides stercoralis	5–9 days	
Trichuris campanula	84 days	
Taenia taeniaeformis	> 35 days	
Circulatory system		
Dirofilaria immitis[*]	220 days	2 days (L3), 20–40 (usually 30) days (L4) 70–120 days (L5)

[*] 100 L3 per cat is usually used for preventive studies. Infections are induced by intra-venous transplantation of 4 pairs of male and female worms about 210 days of age. Infections induced by sub-cutaneous inoculation of infective L3 should not be used. Microfilaremia, even if present, usually lasts only 1–2 months, so treatment for microfilariae is not needed in cats. Sub-cutaneous induced infections of L3 are not recommended for cats.

knowledge of the biology and life cycles of the targeted parasite(s).

Studies regarding *Echinococcus* spp. and *Dirofilaria* spp. are usually conducted with induced infections, due to zoonotic risk considerations for echinococcosis and the complexity of the claims for heartworm. Due to the zoonotic potential of *Echinococcus* spp., studies should be carried out under appropriate biosafety conditions.

For parasite species with a low prevalence and/or the difficulty to find naturally infected hosts, only experimental models may be available. For helminths infecting domestic carnivores, induced infections may be the only method to determine effectiveness of a product because of difficulties in recruiting enough infected animals in the field. This includes *Filaroides milksi*, *F. hirthi, Oslerus osleri, Crenosoma vulpis, Spirocerca lupi, Physaloptera* spp., *Mesocestoides* spp., *Dibothriocephalus* spp., and *Spirometra* spp. The list is not exhaustive.

On the other hand, in the absence of experimental models, field efficacy studies may be the only available option to assess efficacy against some helminths, such as *Capillaria aerophila (syn Eucoleus aerophilus), C. boehmi (syn. E. boehmi), C. plica (syn. Pearsonema plica)* (Moravec, 1982), *Thelazia callipaeda,* or *Dioctophyme renale.*

3.2. Efficacy assessment in experimental infection models, including adequacy of infection

The general principle of efficacy assessment in experimental studies is to compare the parasite counts after necropsy, between treated group (s) and untreated control group. A reduction of at least 90 % in worm counts is in general required to demonstrate sufficient efficacy (Geurden et al., 2022). An efficacy of 100 % may be requested for some dog and cat parasites due to their pathogenicity in the hosts (such as *D. immitis)*, or due to their zoonotic risk (such as *Echinococcus granulosus* or *E. multilocularis)*.

Furthermore, a statistically significant difference in parasite counts between treated and untreated groups is required (Geurden et al., 2022), and the number of infected dogs or cats in the untreated group should be adequate to allow statistical comparison (Zhao et al., 2021). For dose determination or dose confirmation studies, it is generally recommended to include at least 6 animals per experimental group (Geurden et al., 2022). In studies with cats and dogs, this number is commonly increased to eight or ten animals per group to ensure a minimum of six adequately infected animals in the control group. Depending on the parasite species under evaluation, the minimum number of helminths to be recovered from control animals to achieve adequate infection should be defined in the study protocol. Final conclusions regarding adequacy of infection should be made based on statistical data, historical data, literature review, or expert testimony. Generally, a minimum of five nematodes in at least six infected control animals is considered an adequate infection. The minimum number may be reduced when targeting a parasite in a host species which is considered less susceptible (i.e., the minimum number of adult *D. immitis* to be recovered in infected control cats; see discussion in section 3.4.).

To demonstrate a significant difference between treated and untreated groups, the type of statistical analysis may vary based on study designs. The distribution of the infection level in a group of animals usually does not follow a normal distribution, but rather is aggregated. For example, when infecting cats with L3 *D. immitis*, it is considered acceptable to obtain an average rate of infection of two or more adult heartworms. A very limited infection rate is to be expected for *E. multilocularis* in cats despite infective challenges with hundreds of protoscolices because cats are less suitable compared to canids (e.g., foxes and dogs). In the case of studies on naturally infected animals the level of infection with worms is not known at the beginning, and in case of studies with induced infections, the success of the infection is not known either. When the parasite numbers are not known and the distribution is aggregated, geometric means could be considered, representing a more central view of the infection rates. In that case, the log-count transformation provides data for a normal distribution, allowing ANOVA test for comparisons of infection rates, but this may not always be the case. Non-parametric tests compare the medians between groups rather than the means (e.g. Wilcoxon sum rank test) and may also be used in the case of non-normal distribution, without any transformation of the parasite counts.

For ethical reasons, the development of alternative approaches to the assessment of parasite burden through in vivo diagnosis instead of post-mortem diagnosis (i.e., after euthanasia and necropsy) should be encouraged. For non-zoonotic gastro-intestinal nematodes, the faecal egg count may be an acceptable criterion to demonstrate efficacy, as it is done in field efficacy studies. “Parasite-free status” (or undetectable parasite status) may also be an acceptable criterion. For this the number of infected animals in the control group versus the number of infected animals in the treated group is compared, not based on parasite count, but on an alternative yet accurate method of diagnosis for the infection (e.g., larval faecal counts through Baermann for lungworm infections, antigen and microfilarial detection for dirofilariosis, coproantigens and PCR for *Echinococcus* spp.). These diagnostic methods are commonly used in field studies using privately owned animals, when necropsy is not possible. It is also related to the fact that in companion animals, like in human medicine, the threshold for treatment is based on the diagnosis of infection and not necessarily on the level of infection, as is often the case in production animals or horses. The assessment of the number of infected animals, based on validated copromicroscopy and/or other diagnostic methods, between treated and untreated animals could become acceptable in the future in experimental in vivo studies as it is in field studies, especially if targeting 90–100 % parasite-free dogs/cats depending the parasite.

3.2.1. Experimental infections with gastro-intestinal helminths

3.2.1.1. Efficacy studies (experimental) against major gastro-intestinal nematodes. The general principle of efficacy assessments in experimental efficacy studies are provided in Fig. 1(i.e. assessment of adulticidal efficacy) and Fig. 2(i.e. assessment of larvicidal efficacy), respectively. Timing between inoculation and treatment depends on the prepatent period of the respective parasites after which copromicroscopic examination will reveal egg shedding. It is generally recommended to perform necropsy seven to ten days after treatment; however, the duration may vary depending on factors such as the pharmacokinetic profile of the active ingredient, or the time for elimination of parasites.

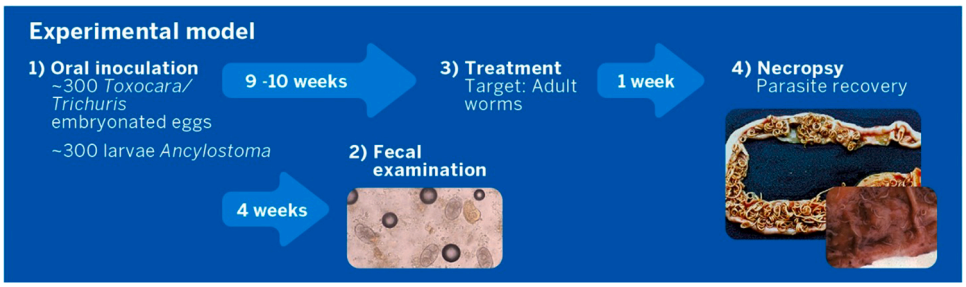

Fig. 1. Assessment of treatment efficacy against adult stages of *Toxocara*, *Trichuris* and *Ancylostoma* using an experimental infection model.

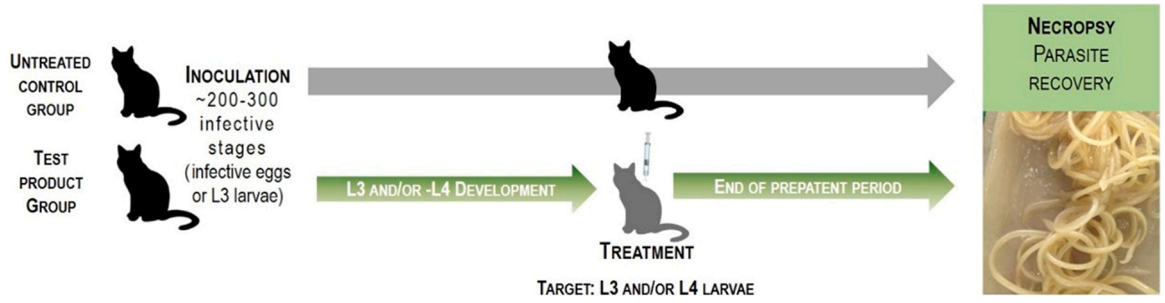

Fig. 2. Assessment of treatment efficacy against immature stages of *Toxocara* using an experimental infection model.

For induced infections, the number of infective stages to be used often remains approximate and may depend on the specific isolate. The actual number of infective stages used for infection should be included in the final report. Tables 3 and 4 show the suggested range of infective stage numbers to induce helminthic infections in dogs or cats, respectively. Inoculating a high number of infective stages at once may trigger acute immune response, or competition between the parasites, finally resulting in reduced infection rates. To simulate infections in natural environments in dogs and cats and to avoid the aforementioned effects, it may be valuable to repeat infections with multiple inocula containing low numbers of infective stages rather than a single dose containing a high number of stages. This has for example been done in experimental efficacy studies assessing activity against *Angiostrongylus vasorum* in dogs (Knaus et al., 2014; Lebon et al., 2016) (see Section 3.2.2.).

As already discussed in Section 3.2., the minimum adequate number of helminths in individual control animals should be defined in the protocol. However, conclusions regarding adequacy of infection will be made as part of the final report based on statistical analysis, historical data, literature review, or expert testimony. As a rule, a minimum of five nematodes in at least six individual control animals can be considered as an adequate infection. This minimum number may be reduced when dealing with a parasite/host interaction known to provide few adult establishments. This might be the case when the host is not the primary natural host, providing a lower susceptibility, e.g., infecting cats with *E. multilocularis* (Section 3.2.1.2.) or *D. immitis* (Section 3.2.3.).

For studies designed to assess the efficacy of an anthelmintic treatment against transmammary transmission of *Toxocara canis* or *T. cati* larvae to puppies/kittens, naturally or artificially infected pregnant females should be treated prior to or just after parturition. The efficacy will be determined by counting larvae in the milk of females, the eggs in faeces, and/or adult worms in the small intestines of their litter if requested by the regulatory agencies.

Details of recommended age for experimental infection per most relevant parasite species are provided in Table 5.

Table 3
Range of infective stage numbers recommended to produce adequate infections of intestinal helminths in dogs for anthelmintic evaluation.

Parasites	Range
Gastrointestinal tract	
Toxocara canis	100 – 500 larvated eggs
Toxascaris leonina	200 – 3000 larvated eggs
Ancylostoma caninum	100 – 300 L3
Ancylostoma braziliense	100 – 300 L3
Uncinaria stenocephala	1000 – 1500 L3
Strongyloides stercoralis	1000–5000 L3
Trichuris vulpis	100 – 500 larvated eggs
Spirocerca lupi	40 – 45 L3
Echinococcus granulosus	10,000–40,000 protoscolices
Echinococcus multilocularis	15,000 protoscolices
Respiratory tract	
Angiostrongylus vasorum	~250 L3

Table 4
Range of infective stage numbers recommended to produce adequate infections in cats for anthelmintic evaluation.

Parasites	Range
Gastrointestinal tract: small intestine	
Toxocara cati	100 – 500 larvated eggs
Toxascaris leonina	200–3000 larvated eggs
Ancylostoma tubaeforme	100 – 300 L3
Ancylostoma braziliense	100 – 300 L3
Strongyloides stercoralis	1000 – 5000 L3
Echinococcus multilocularis	10000 – 30000 protoscolesces
Gastrointestinal tract: large intestine	
Trichuris campanula	100 – 500 larvated eggs
Respiratory tract	
Aelurostrongylus abstrusus	~225 L3

Table 5
Recommended age of cats/dogs to produce adequate experimental infection.

Parasites	Recommended age
Toxocara canis; T. cati; Toxascaris leonina	Less than 16 weeks
Toxascaris leonina	12 weeks to 2 years, but possible with older animals
Ancylostoma tubaeforme; A. braziliense; A. ceylanicum; A. caninum	Less than 16 weeks, but possible with older animals
Uncinaria stenocephala	Less than 12 months
Strongyloides stercoralis	Less than 6 months

3.2.1.2. Efficacy studies against cestodes. Efficacy studies against cestodes have historically been based on the recruitment of naturally infected dogs or cats (see Section 3.2.2.). Experimental infections are used to assess the cestodicidal efficacy against *Echinococcus* spp., and may also be used for *Dipylidium caninum* or *Taenia* spp.

The *Dipylidium* model is based on the acquisition of infected *Ctenocephalides* fleas to allow for the infection of dogs and cats (Pugh and Moorhouse, 1985; Pugh, 1987). In recent experimental *Dipylidium* infection studies, the batches of infected fleas had a rate of infection of 10–35 %, and dogs and cats were infested with 100–200 fleas from these batches (Beugnet et al., 2013; Beugnet at al., 2017). Adult worms were obtained within 5–6 weeks allowing to assess efficacy of anthelmintic drugs (Charles et al., 2005; Schroeder et al., 2009; Knaus et al., 2021). It has been demonstrated recently that cats and dogs are not infected with the same *D. caninum* genotypes (Beugnet et al., 2018; Labuschagne et al., 2018). It is thus important to use the dog genotype for efficacy studies in dogs, and the feline genotype for efficacy studies in cats, to generate relevant efficacy data.

Concerning *Echinococcus* spp., studies need to be conducted with the adequate level of biosafety due to the zoonotic potential of the cestodes' eggs. The protoscolices are extracted from an intermediate host previously infected. *E. granulosus* protoscolices are usually extracted from infected sheep. *E. multilocularis* protoscolices are extracted from the livers of infected mice, rats or cotton rats. A range of 10,000–40,000 protoscolices are used to be inoculated orally per dog to produce infections with *E. granulosus* or *E. multilocularis* (Andersen et al., 1985; Andersen et al., 1979; Gemmell et al., 1980), or per cat to produce infections with *E. multilocularis* (Andersen et al., 1981; Jenkins and Romig, 2000; Prullage et al., 2021). As for nematodes, the efficacy assessment against immature stages is based on drug administration before the end of the prepatent period (4–5 weeks), whereas efficacy assessment against adult stages is based on drug administration after the end of the prepatent period.

3.2.2. Efficacy studies against metastrongyloid lungworms

Lungworms tend to be more and more commonly diagnosed in dogs (especially angiostrongylosis due to *Angiostrongylus vasorum*) and cats (especially aelurostrongylosis due to *Aelurostrongylus abstrusus*, and troglostrongylosis due to *Troglostrongylys brevior*). Treatments against lungworms are mostly aimed to prevent the establishment of adult worms in the pulmonary arteries, bronchioles and/or alveolar ducts, or to treat infections with clinical manifestations. Experimental infection models have been developed to assess the preventive or therapeutic efficacy against these metastrongyloid nematodes. These experimental infection models rely on naturally infected dogs and cats that serve as donors of L1 larvae for infecting terrestrial snails, mainly *Helix aspersa*. Once larvae have developed to L3, infected snails are artificially digested to release the infective L3, which are washed and then used to inoculate cats or dogs *per os*. Preventive and therapeutic efficacy studies against lungworm in dogs or cats can be conducted by inoculating animals with approximately 250 L3 of *A. vasorum* (Table 3) or approximately 225 L3 of *A. abstrusus* (Table 4) (Knaus et al., 2014; Lebon et al., 2016; Becskei et al., 2020; Heuer et al., 2020; Gianelli et al., 2015). For example, cats can be experimentally infected with *A. abstrusus* and treated four days post infection to target third-stage larvae, treated seven days post infection to target fourth-stage larvae, treated 14 days post infection to target immature adults or treated 32 days post infection (after confirmation of shedding of larvae) to target adult worms (Knaus et al., 2015).

3.2.3. Efficacy studies against Dirofilaria immitis

The classical design applied for heartworm preventive efficacy studies with monthly treatments is based on the inoculation of infective L3 (30–100 L3 extracted from mosquitoes) followed by a larvicidal treatment 30 days later (Fig. 4) (Donahoe, 1975; Chandrashekar et al., 2014). The aim is to demonstrate a killing effect against immature stages within one month after infection, thus preventing the establishment of adult worms in the arteries and right ventricle of the heart. When seeking an indication based on one, two, three or four monthly doses, necropsy for efficacy evaluation should be done five months after infection. For an indication including five or more monthly doses requires the extension of the usual five months waiting period to allow at least one month between the last treatment dose and necropsy.

For long-lasting or extended-release formulations (>6 months), the design is modified to prior treatment followed by the inoculation of infective L3. This design aims to evaluate the efficacy against infective L3 at the time of their transmission by the mosquitoes (e.g., 6–12 months for moxidectin injectable formulations, 2 months for moxidectin topical formulations). For such studies necropsy is performed five months after last infection.

To overcome the variability of infection rates in experimental infections, and to be able to assess curative efficacy or safety in heartworm positive animals, the study design can be designed based on the transplantation of adult worms (5−15) from an infected donor dog to receiver cats or dogs (Rawlings and McCall, 1985; Savadelis et al., 2017; Savadelis et al., 2020).

Unlike in dogs, there is considerable variation in the number of adult worms recovered from heartworm infected cats with many cats harboring up to one worm. Naturally infected cats are frequently found to be infected with only a single worm, which created significant pathology and frequently lead to the death of this host species. From the early 1970 s until the late 1990's, the UGA/TRS *D. immitis* isolate was used in most feline heartworm prevention studies conducted in laboratories in the USA (McTier et al., 1992a,b; Stewart et al., 1992; McTier et al., 2000; Arther et al., 2003; Genchi et al., 2004). Although this isolate was randomly selected in the early 1970s, cats were found to harbor low-level, but reasonably predictable, infections. Accordingly, the US Food and Drug Administration (FDA) required that at least 60 % of infected control cats be infected with at least two heartworms per animal for a study to be considered valid. Due to the emergence of macrocyclic lactone resistance in heartworms in the late 1990s, the FDA limited the use of an isolate to five years. Subsequently, numerous isolates have been used since then, with more variability and less predictable infection levels (Baker et al., 2014; McTier et al., 2019; Baker et al., 2021). We recently reviewed adult heartworm recovery data on untreated control cats in 16 different laboratory studies conducted over the past two decades (McCall et al., unpublished data). The percentage of infected cats per group (infection rate) ranged from 28.6 % to 100 %. The arithmetic mean number of worms per infected cat ranged from 1.0 to 10.4. Based on the data from these cats and a group size of 10, we propose that a valid study would have at least 6 (60 %) infected cats and the infected cats would have a mean of three or more worms per cat, following inoculation of 100 L3. Using these criteria, 13 of the 16 studies (83.3 %) would be considered valid. The three invalid studies had infection rates of 28.6 %, 55.6 % and 83.3 % and mean numbers of worms per infected cat of 4.5, 1.8 and 1.0, respectively. This proposed criteria better reflects infections with only one or two worms (and rarely more than six) in most cats with naturally acquired infections, and the less predictable experimentally induced infections seen in laboratory cats in the past two decades.

Since an efficacy of 100 % is recommended due to pathogenicity in the host, necropsy is required for all animals in at least one of the required dose confirmation studies. In a second dose confirmation study, efficacy evaluation based on serology against circulating antigen or detection of microfilariae could be considered to reduce necropsy to those dogs which tested positive.

The requirement for 100 % efficacy to claim heartworm efficacy has historically been driven by several studies on the evaluation of heartworm preventive drugs that all yielded an observed efficacy of 100 % with a single dose, using a limited number of susceptible isolates. Recently, the value of a 100 % observed efficacy threshold has been questioned (Vidyashankar et al., 2017), because 1) an insufficient

number of heartworm isolates (strains/biotypes) has been tested in these historical studies, 2) even within a heartworm isolate the susceptibility phenotype can change over time, 3) the establishment rate after an induced infection can significantly impact the efficacy evaluation (Bourguinat et al., 2017). One could perform additional simulations and come up with a lower 95 % Confidence Interval (CI) that would be required to ensure that the true efficacy of a drug meets at a minimum, a very high standard, without requiring the 100 % standard, which is not verifiable (Vidyashankar et al., 2017). More research and discussion are required to reach a consensus whether the strict 100 % (observed) efficacy threshold should be maintained or whether alternative modelling can help in evaluating the true efficacy against third and fourth stage larvae of *D. immitis*. It should also be discussed whether efficacy should be established against susceptible and resistant isolates separately, resulting in separate claims added to the product label.

3.2.4. *Efficacy studies against other nematode infections (e.g. Spirocerca lupi, Dirofilaria repens)*

Anthelmintic studies can be designed to evaluate efficacy against any species of helminths with the necessity to be able to recruit naturally infected animals and/or to be able to do experimental infections. The applied diagnostic methods should reliably confirm infection. With the variability related to the use of in-house tests, having an unknown sensitivity of specificity compared to other tests done by other research groups, it is necessary to describe the applied diagnostic tests concisely in the protocol and to address issues related to their sensitivity and specificity in the final report of a study.

These studies may target emerging parasites, or parasites which are endemic in certain geographies.

S. lupi (oesophagal worm), which mainly infect dogs is highly pathogenic and endemic in several countries having a warm climate from eastern to southern Africa, Middle East, Israel, India, and the Indian Ocean Islands like Madagascar or La Réunion. It is sporadically imported in Europe. Experimental models of infection have been developed and are used to demonstrate the curative efficacy against adult oesophageal worms, but also the preventive efficacy of adult establishment through larvicidal efficacy (Kelly et al., 2008; Kok et al., 2010; Kok et al., 2011; Le Sueur et al., 2010; Beugnet et al., 2016). One major biological threat is that the infective L3 reach the wall of the caudal thoracic aorta via the gastric arteries within 10 days after infection and will stay and develop to immature worms around 90–110 days in this location before reaching the oesophagus to become adults. Thus, a highly efficacious preventative anthelmintic should be able to maintain a larvicidal killing effect to prevent aortic lesions. Short acting larvicidal anthelmintic formulations will kill larvae and prevent oesophageal nodules, but will not avoid the aortic lesions, despite regular (i.e., monthly) administrations (Austin et al., 2013; Beugnet et al., 2016).

An experimental model has recently been developed for *D. repens*, the zoonotic agent of subcutaneous dirofilariosis in dogs (Ciuca et al., 2020). As for *D. immitis*, it first requires at least one infected donor on which mosquito vectors are fed and then incubated to let the microfilariae develop to the infective L3 stage, and finally to dissect the mosquitoes to recover the L3 for infection. Dogs can then be inoculated subcutaneously with 50 L3. The model showed 80 % infection rate in dogs. Skin nodules and microfilariae were observed on Day 220, which can be considered as the pre-patent period. The sub-cutaneous adult worms were recovered after 10 months through necropsy by removing the full skin and inspecting the sub-cutaneous tissue all over the body. The adult worms were recovered in several places and not only at the larval injection site.

An example for a parasite where control would benefit from an experimental infection model is *Dioctophyme renale* (syn. *Dioctophyma renale*), which is primarily prevalent in South America. This parasite resides in the kidney pelvis and is endemic in several countries, for example in Argentina. Currently the only option for treatment is surgical removal of the worms, and sometimes the removal of the infected kidney. Studies demonstrating larvicidal activity and prevention of the establishment of this giant nematode in the kidney bassinet of dogs would be of interest for improved control of this infection.

Similarly, having experimental models for *Thelazia* spp. and *Capillaria* spp. (including *Capillaria aerophila* and *Capillaria plica*) would be beneficial to assess and differentiate adulticidal and larvicidal efficacies of anthelmintics.

For many parasitic helminths, the only actual possibility to assess efficacy of anthelmintic treatment is the conduct of field studies, as is the case for *Thelazia* spp. and *Capillaria* spp. (see Section 4).

3.3. *Natural infections*

The general study design for natural infections is provided in Fig. 3. Naturally infected animals are selected based on eggs/larvae detection or expelled proglottids of helminths (including gastrointestinal parasite or parasite of other locations with excretion of eggs or larvae in faeces), or other parasitological and/or immunological methods (e.g. *D. immitis*).

The natural infection models allow to assess efficacy against any species of helminths, including those for which no experimental infection models are established, like for *C. plica* in cats (Knaus et al., 2014).

Studies on naturally infected animals can generate relevant efficacy data for adult helminths, based on the diagnosis of patent infections, but do not allow to assess larvicidal activity of anthelmintics.

4. Field studies

Field efficacy studies are conducted primarily to confirm the efficacy and safety of the final formulation of the anthelmintic when used at the intended dose under natural conditions in a representative population of dogs or cats (e.g. privately owned pets). This may include animals of different physiological status, breed, and/or age group within the targeted host population. Efficacy in field studies cannot be determined by comparing parasite burden by necropsy between groups. Anthelmintic efficacy is typically evaluated based on biomarkers of worm infection, such as faecal egg or larval counts.

The general principles on how to conduct field studies are described in detail in Geurden et al. (2022). Field efficacy studies should be conducted as multicentre studies in at least two different geographical regions in which the parasites naturally occur. These regions should reflect different epidemiological conditions to follow the concept to include genetically diverse parasite populations.

In field studies, study animals in both the control group and the treated group shall be equally exposed to natural infections. Except for the treatment, the control animals should be handled and evaluated in the same manner as the treated animals. While a minimum of 25 % of the number of animals (selected at random) in the treated group has previously been advocated for the size of the control group (Wood et al., 1995; Vercruysse et al., 2001; Hennessey et al., 2006), it is recommended to discuss the required number of animals in the control group versus the treated group(s) with a statistician to find an adequate ratio for the design of the individual field study.

If animal welfare and veterinary medical considerations allow, an untreated or negative control group may be used, and exposure to infection should be confirmed in these untreated animals at the same time-points as for the treated group(s). If not, the efficacy of the product should be evaluated against an approved reference product (active/positive control). When a positive control group is used, it is recommended to generate sufficient evidence that the animals in the field study were indeed infected prior to treatment (for example through faecal eggs/larvae detection) or exposed to infection after treatment, as appropriate. As discussed in above paragraphs, counting eggs/larvae pre and post treatments within treated groups, or post-treatment between positive group and negative control group is generally requested by registration agencies to confirm the efficacy of anthelmintics and grant a claim. But considering that every infected companion animal will be treated, it would be acceptable to also include the criterion parasite-free

Fig. 3. Assessment of anthelmintic efficacy using a natural infection model. 1 week = 7–10 days.

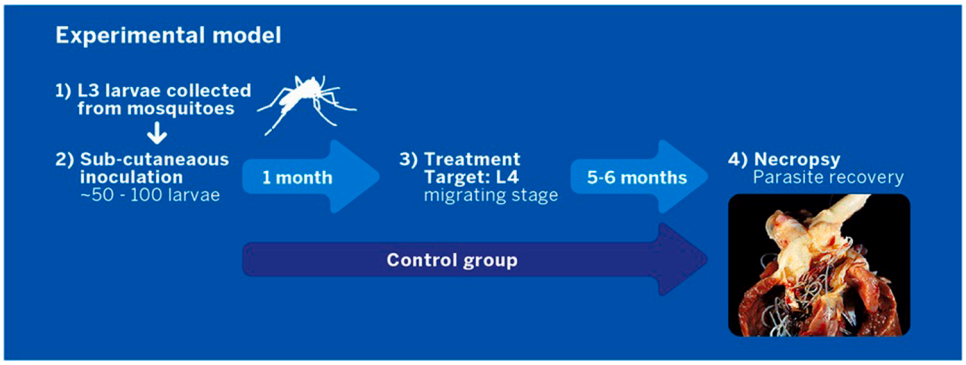

Fig. 4. Study design to assess larvicidal efficacy against *Dirofilaria immitis*.

post-treatment. Exposure to infection during the field study can be documented through recent and historical epidemiological data.

The targeted parasites in field studies are mainly the major intestinal parasites, e.g. roundworms (*Toxocara* spp., *Toxascaris leonina*), hookworms (*Ancylostoma* spp., *Uncinaria stenocephala*), whipworms (*Trichuris vulpis*), and heartworm (*D. immitis*), but field studies are also built to assess the efficacy of anthelmintics against other internal helminths for which no experimental models exist yet. This is for example the case for the capillarid infections, i.e. *C. plica*, *C. aerophila* and *C. boehmi* (Traversa et al. 2012; Knaus et al., 2014; Rehbein et al., 2014; Veronesi et al., 2014; Veronesi et al., 2017; Di Cesare et al. 2017; Di Cesare et al., 2021). It is yet the only possibility to assess either the curative or the preventive efficacy against the eyeworm *Thelazia callipaeda* which is endemic in Asia and currently spreading in Europe (Bianciardi and Otranto, 2005; Motta et al., 2012; Lechat et al., 2015; Lebon et al., 2019;; Zanet et al., 2021).

5. Conclusions

The WAAVP anthelmintic guidelines are revised to ensure consistency over the different host animal species and considering recent scientific advancements. The present species-specific anthelmintic guidelines provide updated and standardized guidance for the conduct of dosage determination, dosage confirmation and field efficacy studies companion animals. It is complementary to the general guidelines (Geurden et al., 2022). The guidelines cannot be exhaustive in all possible study designs but are giving indications on the way to build a protocol and conduct an efficacy study. These studies will evolve in parallel to the development of new experimental models and new diagnostic tools.

CRediT authorship contribution statement

All authors contributed equally to conceptualization, investigation and writing (original draft). Fred Beugnet was responsible for the supervision of the initiative.

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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