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An in vitro larval motility assay characterizes anthelmintic efficacy against *Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus*

Haifaa A. Mahjoub, BSc, MSc, PhD[1]; Daniela Bedenice, Dr med vet, Diplomate ACVIM (LA), Diplomate ACVECC (Eq)[2]*; Henrik Stryhn, MSc, PhD[3]; Jenny Yu, MSc[3]; Spencer J. Greenwood, BSc, MSc, PhD, DVM[4]; Gary A. Conboy, BSc, DVM, PhD, Diplomate ACVM[5]

[1]Biological Sciences Department, College of Sciences & Arts, King Abdulaziz University, Rabigh 21911, Saudi Arabia
[2]Department of Clinical Sciences, Tufts Cummings School of Veterinary Medicine, North Grafton, MA
[3]Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada
[4]Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada
[5]Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada

*Corresponding author: daniela.bedenice@tufts.edu

OBJECTIVE
This study determined the in vitro efficacy of 6 common anthelmintics (eprinomectin, ivermectin, milbemycin oxime, moxidectin, selamectin, and fenbendazole) on motility (viability) of infectious third-stage larvae (L_3) of *Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus*, which are important causes of canine and feline cardiopulmonary disease.

SAMPLES
First-stage larvae (L_1) from *C vulpis*, *An vasorum*, and *Ae abstrusus.*

PROCEDURES
Naïve *Limax maximus* slugs were fed 1,000 to 2,000 L_1 and held at 16 °C for at least 4 weeks to produce live L_3. Approximately 50 to 100 L_3/well were subsequently incubated in culture media alone or media containing 6 separate test anthelmintics at 4 concentrations, to bracket expected in vivo drug plasma levels in anthelmintic-treated dogs and cats. Drug effects on L_3 motility (viability) were analyzed by multilevel logistic models, generating dose-response relationships. Experiments were completed 1-9/2019.

RESULTS
Drug concentration estimates corresponding to a 50% larval mortality rate identified that *C vulpis* was the most sensitive species to the anthelmintics tested. *Ae abstrusus* was most susceptible to moxidectin and selamectin, while *An vasorum* was insusceptible to all anthelmintics tested, except for selamectin at high drug concentrations.

CLINICAL RELEVANCE
The in vitro anthelmintic response to antiparasitic agents may guide and improve disease therapy and prevention. Considering the observed lack of efficacy against L_3, monthly anthelmintic treatment for protection against *An vasorum* infection in dogs would primarily rely on the anthelmintic's adulticidal activity. Maximal preventive control for *An vasorum* would, therefore, require at least 1 treatment administered a minimum of 1 week after the end of the transmission season.

Keywords: *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Aelurostrongylus abstrusus*, antilarval, antiparasitic

The metastrongyloids, *Aelurostrongylus abstrusus*, *Angiostrongylus vasorum*, and *Crenosoma vulpis* are important causative agents in canine and feline respiratory and cardiopulmonary diseases in parts of North America and Europe, posing a significant health risk to companion animals. Pets acquire infection by the ingestion of infective third-stage larvae (L_3) contained in the tissues of gastropod intermediate or paratenic hosts, and the L_3 then migrates to the heart and lungs via lymphatic vessels or the hepatic portal system and matures into the adult stage.[1] The adult stage resides in the cardiorespiratory system of infected animals; for example, *C vulpis* is found in the bronchi, bronchioles, and trachea, while *Ae abstrusus* resides in the lung

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parenchyma and alveolar ducts. *An vasorum* is found in the pulmonary artery and the right ventricle. The prepatent period (PPP) of *C vulpis* is 19 to 21 days and the PPP of *Ae abstrusus* is about 5 to 6 weeks. For *An vasorum*, the PPP is 28 to 108 days.[1] After the end of the PPP, L_1 are coughed up into the pharynx and then swallowed again, after which they pass through the stomach into the small intestine, and are released via feces into the environment.[1] Lungworm disease is primarily treated by administering anthelmintics that eliminate adult lungworms, minimize larval fecal shedding, and decrease lung damage. Previous studies indicate that certain benzimidazoles (BZD) and macrocyclic lactones (MLs) are effective for the treatment of crenosomosis, angiostrongylosis, and aelurostrongylosis.[2–8] While ML products are formulated for oral, topical, or injectable administration, and are approved for heartworm prevention in dogs or cats, little is currently known about the minimum drug concentrations necessary to establish treatment efficacy and disrupt larval development in animals infected with metastrongyloid lungworms.

Several previous studies have evaluated the in vitro activity of anthelmintic drugs against various helminths (including *Haemonchus contortus*, *Strongyloides* spp, *Trichuris* spp, *Oesophagostomum dentatum*, *Ancylostoma caninum*, *Caenorhabditis elegans*, human hookworms and trichostrongyloids) using larval development and motility assays.[9–16] The purpose of this study was to compare the efficacy of select anthelmintics against immature stages (L_3) of various lungworm species that affect dogs and cats, using an in vitro larval motility assay. We hypothesized that the chosen anthelmintics would decrease larval viability in a dose-dependent manner, in conjunction with their known effect on adult worms. Eprinomectin, milbemycin oxime, and moxidectin have been reported as effective in use against *Ae abstrusus* or *An vasorum* as monthly treatments, similar to commonly used heartworm preventives.[16–19] Determining whether these or other anthelmintics have efficacy against the early migrating stages of the parasite or are only effective against adult stages has implications for designing a control program to achieve optimal and efficient control.

Materials and Methods

Parasites

First-stage larvae of *Ae abstrusus* and *An vasorum* was recovered from feces of naturally or experimentally infected cats and dogs, respectively, by a quantitative Baermann examination.[20] In brief, 12 g of feces were wrapped in a double layer of cheesecloth, placed in a glass Baermann funnel containing warm water, and left overnight (>12 h). Water (50 mL) was drawn from the funnel into screw-top plastic graduated centrifuged tubes. Samples were centrifuged (700 g) for 10 minutes, the supernatant discarded; the volume was adjusted to a total of 5 mL, and the pellet re-suspended by vortexing for 20 seconds. The L_1 concentration was determined by counting all of the larvae in 50 µL subsamples placed on a slide with a coverslip and examined using a compound microscope under a 10X objective. Morphological and morphometric verification of L_1 was performed for each positive sample.[21]

Aelurostrongylus abstrusus L_1 were isolated from the feces of a naturally infected domestic cat from Newfoundland-Labrador, Canada. Larvae were recovered by the Baermann technique, identified, and enumerated.[20]

Angiostrongylus vasorum L_1 were recovered from the feces of an experimentally infected purpose-bred research beagle (Marshall BioResources) housed at the Atlantic Veterinary College (original source: naturally infected red foxes from Newfoundland-Labrador, Canada). Larvae were recovered by the Baermann technique, identified, and enumerated.[20] The L_1 recovered from the feces of this dog consisted of a mixture of 95% to 98% *An vasorum* and 2% to 5% *C vulpis*.

Crenosoma vulpis L_1 were acquired by dissection of the lungs at necropsy of naturally infected red foxes (*Vulpes vulpes*) harvested for fur by trappers on Prince Edward Island, Canada. The fox carcasses were frozen at −20 °C for 5 to 7 months before necropsy. Adult *C vulpis* were recovered by lung flush,[22] and female worms were placed in a Petri-dish (60 mm diameter X 15 mm) and cut into pieces using a razor blade. Female worm fragments were placed in cheesecloth in a Baermann apparatus, L_1 were recovered, identified, and enumerated.[20]

Gastropod exposure

Naïve laboratory raised slugs (*Limax maximus*) were infected using L_1 recovered by the Baermann technique for each lungworm species (*C vulpis*, *An vasorum*, and *Ae abstrusus*). Two-hundred microliters of L_1 solution (containing 1,500 to 2,000 L_1) was vortexed for 20 seconds, then placed on top of romaine lettuce in 6-well microtiter culture plates (12.5 cm X 8.5 cm X 2 cm). Slugs were placed in separate test wells containing L_1 and lettuce, and the culture plate was incubated at 16 °C and 70% relative humidity (RH). After the slugs consumed the majority of the lettuce, they were transferred to individual plastic lock-top food storage containers with a damp paper towel on the bottom, fed fresh romaine lettuce and maintained in an incubator at 16 °C and 70% RH.

Gastropod digestion—At least 4 weeks post-infection, L_3 were recovered by artificial digestion of slugs in pepsin-hydrochloric acid (HCl) solution (0.3 g pepsin, 0.4 mL concentrated HCl, 50 mL distilled water per each slug). Gastropods were decapitated and placed in a double layer of cheesecloth and suspended in the digest solution in 50-mL screw-top centrifuge tubes for 2 h at 37 °C. The remnants of the slugs were discarded, and the number of L_3 counted and used in the assay. Third-stage larvae were identified based on caudal end morphology; a *C vulpis* caudal end terminates in a simple point, whereas *An vasorum* and *Ae abstrusus* larvae have a rounded knob at the terminal caudal end.[20]

In vitro larval motility assay—The methodology was adapted from the motility assay described for

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hookworm and *Strongyloides* spp,[12] and from a previously described assay measuring the effects of avermectins on the motility of L_3 of the ruminant gut parasite *Haemonchus contortus*.[23] In the current study, 24 well-plates were used.

Drug solutions—Eprinomectin, ivermectin, milbemycin oxime, moxidectin, selamectin, and fenbendazole were purchased from Sigma-Aldrich and Cayman Chemical Co, and stock solutions of each drug were prepared in 0.1% dimethyl sulfoxide (DMSO) **(Table 1)**. The final drug concentrations in the assay plate wells consisted of a series of dilutions starting at 0.5 ng/mL for eprinomectin, 0.5 ng/mL for ivermectin, 1 ng/mL for milbemycin oxime, 5 ng/mL for moxidectin, 1 ng/mL for selamectin, and 1 ng/mL for fenbendazole (using duplicate wells of each drug concentration/plate, 3 replicates of each plate/experimental treatment, and triplicate experiments for each species on different days) **(Figure 1)**.

Table 1—Anthelmintic efficacy against *Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus* was determined using an in vitro larval motility assay.

	Dosage used (ng/mL)			
Drugs	Low	Medium	High	Overdose
Eprinomectin	0.5	20	200	400
Ivermectin	0.5	20	500	1,000
Milbemycin oxime	1	20	1,000	2,000
Moxidectin	5	20	5,000	10,000
Selamectin	1	20	20,000	40,000
Fenbendazole	1	20	1,000	2,000

Approximately 50 to 100 L_3 larvae/well were incubated at 16 °C for 3 days in culture media alone or media containing 6 separate test anthelmintics at 4 concentrations each. Anthelmintic drugs with diverse concentrations were chosen to bracket the expected in vivo drug plasma concentrations in anthelmintic-treated dogs and cats. All experiments were conducted between January and September 2019.

Drug concentrations were chosen based on available pharmacokinetic (PK) data to bracket the expected in vivo drug plasma concentrations in anthelmintic-treated dogs and cats (Table 1). Low and medium drug test concentrations were based on published ranges of maximum plasma concentration (C_{max}) and elimination half-lives, after parenteral or oral administration of clinically relevant anthelmintic doses in dogs and cats.[24–31] Third-stage larvae with an LC50 (drug concentration expected to kill 50% of larvae) at or below these 2 test concentrations may have the highest probability of being effectively targeted using standard clinical dosing. In contrast, high and over-dose concentrations represented sequentially higher drug levels that are less likely achieved in the clinical setting, based on currently investigated routine dose regimens.

Media preparation—Each of the anthelmintic concentrations detailed above was added to RPMI-1640 (cell media) obtained from GE Healthcare Life Sciences, to perform larval motility assays. Control wells contained RPMI alone and RPMI with 0.1% DMSO (Figure 1). Pilot experiments were undertaken to determine the effect of maintaining third-stage larvae of *C vulpis* and *An vasorum* for up to 4 days in RPMI, and to assess the impact of temperature variation on larvae during the incubation period (at 16 °C and 37 °C).

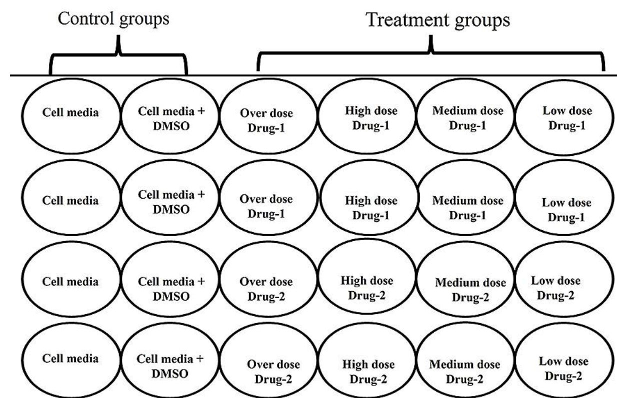

Figure 1—Displays the study's 24-well plate setup to determine the anthelmintic efficacy against *Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus* using an in vitro larval motility assay. and Each test well contained 900 µL of culture media, 100 µL of L_3 solution, and 4 µL of the drug dilution (consisting of 6 separate test anthelmintics at 4 concentrations each); except for the control groups, which contained no drugs dilutions. Three plates were set-up as shown and evaluated for each parasite. Experimental trials occurred between January and September 2019.

Motility assay

Approximately 50 to 100 L_3 (in 100 µL of water, depending on the larvae numbers available on each experimental day) were added to each well of a 24-well plate containing RPMI-1640 and a specific anthelmintic, as described above. Each drug concentration was tested in duplicate on each plate, and the assay was repeated in triplicate in separate experiments. Two control wells were included for each drug treatment: (1) RPMI alone and (2) RPMI with 0.1% DMSO (Figure 1). Plates were incubated at 16 °C for 72 hours. After incubation, 400 to 500 µL of media from each well was removed. Each well was scored by counting the number of larvae showing movement (sinusoidal motion) using a stereomicroscope at 6.3X magnification after adding 400 to 500 µL of the warm digest solution to stimulate larval movement.[20] Larvae that did not display any movement were considered dead. Each test well contained 900 µL of culture media (RPMI-1640), 100 µL of L_3 solution, and 4 µL of the drug dilution except the control groups (no drug dilutions were added) (Figure 1).

It is important to note the presence of a slightly mixed infection of larvae recovered from slugs infected with *An vasorum*, where a small percentage of *C vulpis* larvae (2% to 5%) were detected after slug digestion. The mixed infection was acquired in the original source, red foxes from Newfoundland-Labrador, Canada.

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Statistical analysis

Data were statistically examined using Stata 16.0 (StataCorp LP). The mortality rate of larvae of 3 different species subjected to 6 different drugs at 4 concentrations was analyzed by multilevel (or mixed) logistic models. Data for each species and drug were analyzed separately. The models had drug concentration as a categorical predictor (or fixed effect) and random effects of the hierarchical levels (wells, plates, and replications) of the experiment. Predicted mortality rate at each concentration, with a 95% confidence interval, was obtained from the logistic models for each species and drug and presented in tabular and graphical form. For each species and drug, pairwise comparisons of the mortality rate at the different concentrations used the Bonferroni method to determine statistical significance at $P < .05$. Estimates of drug concentration corresponding to a 50% larval mortality rate were computed from similar mixed logistic models where additionally the drug concentration was assumed to be linearly associated with the mortality rate expressed on logistic scale.

Results

Assay validation

A set of pilot tests for motility assessment was developed and validated using L_3 of *C vulpis* and *An vasorum*. Larvae maintained in culture media for up to 4 days at 16 °C provided a higher survival rate (100%) compared to larvae at 37 °C (79%) (data not shown).

The L_3 of *C vulpis*, *An vasorum*, and *Ae abstrusus* were considered motile if they moved in a sinusoidal motion when stimulated by warm digest solution. The pattern of motion differed between the control groups and the drug exposed groups, where the control group larvae showed rapid, vigorous movement, whereas the drug-exposed larvae moved with a slower, undulating motion. The appearance of drug-affected nonmotile L_3 differed dependent on the species and the 2 drug classes tested. For all larval species tested, as the drug concentration increased, a marked and rapid change from motile to nonmotile L_3 was observed. The nonmotile larvae were considered dead. After 3 days of incubation, even untreated L_3 in the control wells showed minimal movement and appeared to have entered a "dormant" state. The amount of rapid twitching vigorous movement was considerably increased immediately after the addition of the warm digest solution to these dormant larvae.

For *C vulpis*, the predicted larval mortality (as estimated by motility) rate in response to drug exposure is summarized **(Table 2** and **Figure 2)**. Eprinomectin had a 36% larval mortality rate at 20 ng/mL with a predicted LC50 of 28.2 ng/mL **(Table 3)**. A 33% larval mortality rate was observed at 20 ng/mL ivermectin with a predicted LC50 of 56.7 ng/mL. Milbemycin oxime achieved a mortality rate of 30% at 20 ng/mL, with a drug concentration of 67 ng/mL required to kill 50% of larvae. However, the L_3 mortality rate of moxidectin reached 83% at 20 ng/mL with a predicted LC50 of 6.7 ng/mL. In contrast,

Table 2—Depicts the predicted larval mortality rate of *Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus* L_3 after exposure to 6 anthelmintics (eprinomectin, ivermectin, milbemycin oxime, moxidectin, selamectin, and fenbendazole) at 4 drug concentrations each.

			Crenosoma vulpis		***Angiostrongylus vasorum***		***Aelurostrongylus abstrusus***	
Drug name	**Conc. (C) ng/mL**	**Log C**	**Predicted mortality**	**95% CI**	**Predicted mortality**	**95% CI**	**Predicted mortality**	**95% CI**
Eprinomectin	0.5	−0.693	0.068	0.025–0.112	0.015	0.006–0.024	0.024	0.001–0.047
	20	2.996	0.362	0.243–0.482	0.107	0.081–0.132	0.253	0.114–0.392
	200	5.298	0.778	0.682–0.874	0.283	0.241–0.325	0.467	0.290–0.643
	400	5.991	0.898	0.841–0.956	0.393	0.348–0.438	0.584	0.411–0.756
Ivermectin	0.5	−0.693	0.042	0.021–0.063	0.016	0.006–0.025	0.070	0.043–0.098
	20	2.996	0.332	0.258–0.405	0.087	0.058–0.115	0.231	0.173–0.289
	500	6.215	0.795	0.737–0.853	0.198	0.149–0.248	0.479	0.403–0.554
	1,000	6.908	0.880	0.839–0.921	0.303	0.242–0.365	0.650	0.580–0.720
Milbemycin oxime	1	0.000	0.008	0.002–0.014	0.053	0.033–0.074	0.093	0.065–0.122
	20	2.996	0.303	0.271–0.334	0.118	0.083–0.153	0.246	0.199–0.293
	1,000	6.908	0.882	0.861–0.904	0.250	0.195–0.306	0.648	0.595–0.701
	2,000	7.601	0.967	0.955–0.979	0.367	0.300–0.433	0.767	0.722–0.811
Moxidectin	5	1.609	0.280	0.223–0.338	0.067	0.040–0.093	0.204	0.119–0.289
	20	2.996	0.831	0.788–0.873	0.134	0.090–0.177	0.385	0.267–0.503
	5,000	8.517	0.969	0.956–0.983	0.332	0.256–0.407	0.737	0.638–0.836
	10,000	9.210	0.982	0.972–0.991	0.441	0.360–0.523	0.896	0.843–0.949
Selamectin	1	0.000	0.016	0.008–0.025	0.128	0.100–0.156	0.072	0.048–0.095
	20	2.996	0.183	0.156–0.210	0.215	0.179–0.251	0.343	0.293–0.393
	20,000	9.903	0.738	0.708–0.769	0.426	0.380–0.473	0.610	0.560–0.659
	40,000	10.6	0.850	0.826v 0.874	0.630	0.586–0.674	0.772	0.731–0.813
Fenbendazole	1	0.000	0.012	−0.001–0.024	0.017	0.008–0.026	0.049	0.026–0.071
	20	2.996	0.129	0.039–0.220	0.106	0.076–0.136	0.152	0.111–0.194
	1,000	6.908	0.268	0.123–0.413	0.284	0.230–0.338	0.339	0.279–0.399
	2,000	7.601	0.568	0.393–0.743	0.360	0.300–0.420	0.529	0.464–0.594

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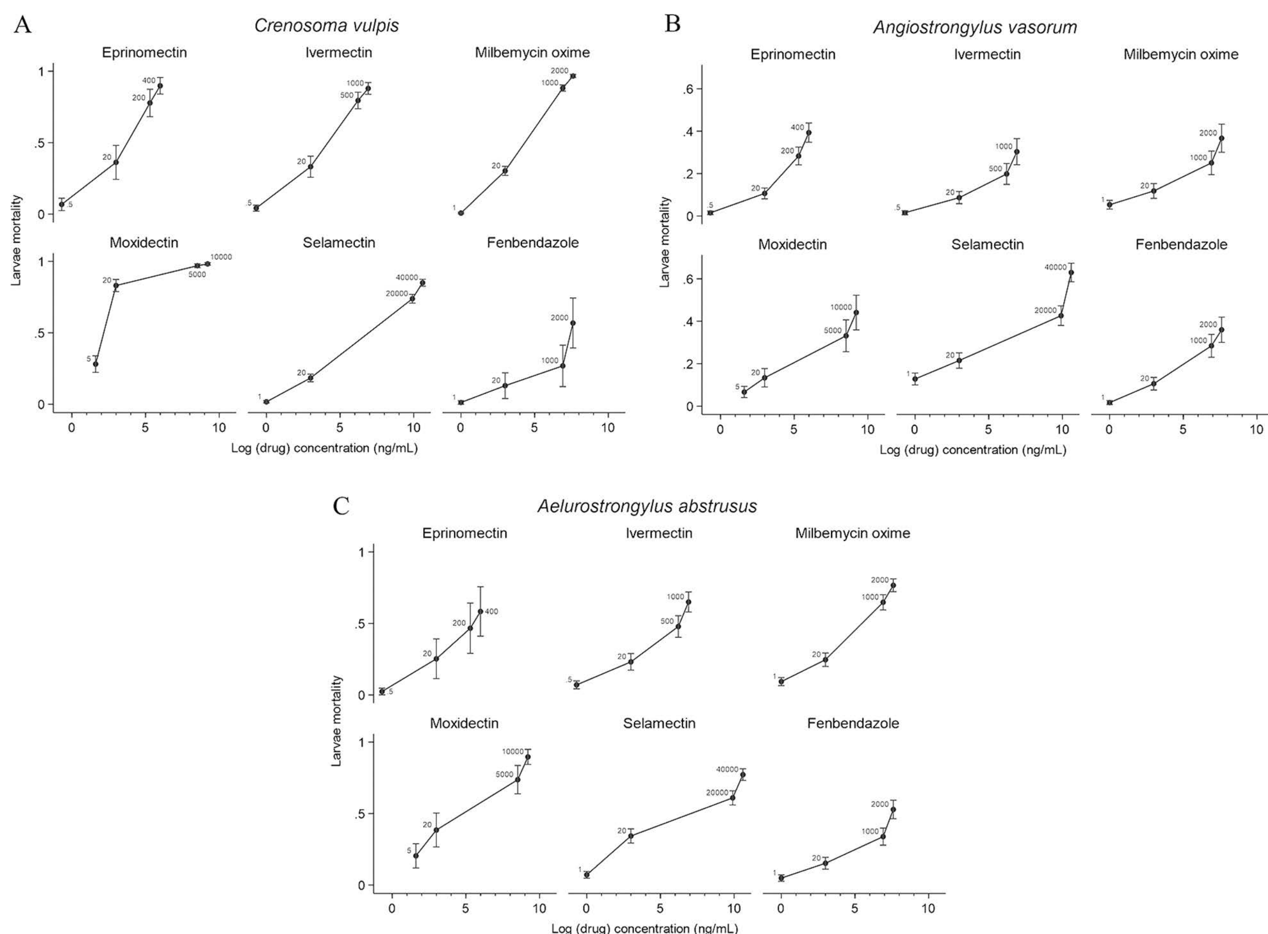

Figure 2—Displays the mortality rate of *Crenosoma vulpis* (A), *Angiostrongylus vasorum* (B), and *Aelurostrongylus abstrusus* (C) third-stage larvae exposed to various anthelmintic drug concentrations. The *y*-axis represents the predicted larvae mortality rate (percent is represented as a fraction where 1 = 100%) and the *x*-axis specifies log concentration of the drug (ng/mL). Numbers within the graph represent the actual drug concentration (ng/mL) with 95% confidence intervals for mortality.

Table 3—Displays the predicted drug concentration (ng/mL) needed to kill 50% of L_3 (LC50) for each species (*Crenosoma vulpis*, *Angiostrongylus vasorum*, and *Aelurostrongylus abstrusus*) and the associated log concentration (Log Conc.).

	Crenosoma vulpis		***Angiostrongylus vasorum***		***Aelurostrongylus abstrusus***	
Drug	**Log Conc.**	**LC50 (95% CI)**	**Log Conc.**	**LC50 (95% CI)**	**Log Conc.**	**LC50 (95% CI)**
Eprinomectin	3.340	28.2 (18.4–43.4)	6.848	943 (658.1 –1350.5)	5.380	217 (57.4–821.3)
Ivermectin	4.037	56.7 (42.4–75.8)	9.273	10,653 (4601.1–24663.1)	5.897	364 (184.5 –718.1)
Milbemycin	4.205	67 (56.9–79)	9.992	21,863 (7716–61945.4)	5.308	202 (133.5 –305.5)
Moxidectin	1.908	6.7 (3.5–13)	10.496	36,172 (10782.6–121342)	4.762	117 (34.3–398.7)
Selamectin	7.063	1,169 (907.5–1505.3)	9.573	14,372 (6547.2–31546.8)	7.083	1,193 (693.5–2050.5)
Fenbendazole	7.788	2,413 (606.1–9604.3)	9.130	9,234 (4636.7–18388.3)	7.921	2,755 (1467.7–5172.1)

20 ng/mL selamectin merely showed an 18% predicted L_3 mortality rate with an estimated concentration of 1,169 ng/mL required to kill 50% of larvae. For fenbendazole, the mortality rate of L_3 larvae was 13% for a 20 ng/mL with a predicted LC50 of 2413 ng/mL. For *C vulpis*, all pairwise mortality rate comparisons among the concentrations within each drug were statistically significant from one another (Table 2).

The mortality rate of *An vasorum* larvae in response to drug exposure is summarized in Table 2 and Figure 2. The drugs eprinomectin, fenbendazole, ivermectin, milbemycin, and moxidectin have a low probability (less than 50%) of killing *An vasorum* L_3 larvae, even at the highest drug concentrations tested, suggesting a lack of drug susceptibility. For selamectin, the estimated concentration needed to kill 50% of larvae was 14,372 ng/mL (Table 3). All pairwise mortality rate comparisons between concentrations of each drug were statistically significant from one another for *An vasorum* (Table 2).

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For *Ae abstrusus*, eprinomectin was most effective at a concentration of 400 ng/mL (Table 2 and Figure 2) with an estimated concentration of 217 ng/mL needed to achieve a 50% larval mortality rate (Table 3). Larval mortality rate at 20 ng/mL ivermectin was 23%, while the estimated LC50 was 364 ng/mL. Similarly, milbemycin oxime achieved a mortality rate of 25% at 20 ng/mL, with a predicted LC50 of 202 ng/mL. The larval mortality rate for 20 ng/mL moxidectin was slightly higher at 38%, with a concentration of 117 ng/mL needed to kill 50% of larvae. Selamectin achieved a comparable mortality rate of 34% at 20 ng/mL, with a predicted LC50 of 1,193 ng/mL. For fenbendazole, the mortality rate of L_3 larvae was only 15% at 20 ng/mL with a predicted LC50 of 2,755 ng/mL. For *Ae abstrusus*, all pairwise mortality rate comparisons among the concentrations within each drug were statistically significant from one another (Table 2).

Discussion

The results of the present study indicate that *C vulpis* is the most sensitive species to the anthelmintic drugs tested, as drug concentrations necessary to achieve a 50% larval mortality rate remained below currently published mean C_{max} concentrations for orally administered ivermectin (0.25 mg/kg), milbemycin oxime (0.25 mg/kg), moxidectin (0.25 mg/kg), and selamectin (24 mg/kg) in dogs.[24–31] *C vulpis*, however, is least sensitive to fenbendazole, since mean reported C_{max} concentrations after oral dosing of 50 and 100 mg/kg in dogs merely reach plasma concentrations of 160 ± 80 ng/mL,[27] and 470 ± 30 ng/mL,[24] respectively, well below the predicted fenbendazole concentration required to kill 50% of larvae in vitro (2413 ng/mL).

Moxidectin and selamectin were the most effective drugs of choice to target infective *Ae abstrusus* larvae, since clinically achievable, maximum plasma concentrations are well above the required drug levels to kill 50% of L_3 (117 ng/mL and 1,193 ng/mL, respectively). More specifically, the reported C_{max} after 24 mg/kg oral selamectin was 10-fold higher in cats (C_{max} = 11,929 ± 5,922 ng/mL) than the required concentration to kill 50% of *Ae abstrusus* larvae in vitro.[25] In contrast, published doses of eprinomectin (0.5 to 2.5 mg/kg topically), fenbendazole (25 to 50 mg/kg orally), ivermectin (0.2 mg/kg topically), and moxidectin (4.3 mg/kg topically or 1 mg/kg orally) are unlikely to achieve therapeutic drug levels against *Ae abstrusus* larvae in cats.

Among the species tested, the larvae of *An vasorum* proved insusceptible to most of the anthelmintics tested. More specifically, the necessary drug concentrations to kill 50% of L_3 larvae were 80-fold, 277-fold, and 155-fold above the achievable C_{max} concentrations in dogs receiving 0.25 mg/kg ivermectin, milbemycin oxime, and moxidectin orally, respectively,[26,27,31] and 20 times above C_{max} in dogs receiving 100 mg/kg oral fenbendazole.[24] Even for selamectin, where concentrations of 14,371.6 ng/mL are predicted to kill 50% of *An vasorum* L_3, the required drug concentration was still twice as high as the reported 7,630 ± 3,140 ng/mL C_{max} achieved in dogs given 24 mg/kg selamectin orally.[25] Similarly, cats merely achieved sub-therapeutic maximum plasma concentrations of 11,929 ± 5,922 (ng/mL) after 24 mg/kg selamectin administered orally.[25] Topical dosing with 2.5 mg/kg eprinomectin in cats is also unreliable, as the reported plasma C_{max} (54.1 ng/mL) is only 1/17 the predicted therapeutic drug concentrations (943 ng/mL).[30] Similarly, 0.2 mg/kg topical ivermectin reaches a mean C_{max} of 16.75 ± 4.04 ng/mL in cats, which is 636 times lower than that necessary to achieve a 50% larval mortality rate based on the current results.

An vasorum can potentially infect cats based on experimental and natural infection studies, but the infections are nonpatent.[32] The current study results indicate a lack of drug efficacy against the L_3 of *An vasorum*. Due to the slight mix of *An vasorum* with up to 5% of the much more susceptible *C vulpis*, the effectiveness of anthelmintics against *An vasorum* in this study was most probably slightly over-estimated. The lack of efficacy was especially concerning as this species appears to have spread at an alarming rate to a wider geographical area. *An vasorum* is very pathogenic and has been considered endemic in Europe, South America (Brazil, Colombia), and in North America (Newfoundland and Labrador in Canada).[1,33] Recent research has indicated the spread of *A vasorum* to West Virginia and Prince Edward Island, Canada.[34,35] The reason for the lack of effect of all of the anthelmintics tested in this study on *A vasorum* and in 4 of the 6 on *Ae abstrusus*, could be attributed to polygenic inheritance since these species belong to the family Angiostrongylidae, while *C vulpis* belongs to the family Crenosomatidae; thus, they might differ in response to treatment. Whether the absence of certain host factors, including immune response, drug absorption, distribution, and metabolism, or differences in drug metabolism between immature stages versus adult worms contribute to therapeutic success needs to be further investigated.

Anthelmintic efficacy was established in vitro for infective L_3 of both *C vulpis* and *Ae abstrusus*, while *An vasorum* larvae demonstrated a lack of drug susceptibility. However, it is also important to note that some partially paralyzed L_3, that still show slight movement, may subsequently die; thus, underestimating drug efficacy at the completion of the motility assay. It is also plausible that longer exposure (> 4 days) to lower drug concentrations may ultimately increase the absolute L_3 mortality rate and improve anthelmintic efficacy.

Results obtained in vitro were interpreted based on reported pharmacokinetic data in dogs and cats, to explore whether commercially available anthelmintics should be investigated as monthly lungworm preventive, in future studies. Since monthly preventives of heartworm infection containing ivermectin most commonly utilizes very low drug doses (0.006 mg/kg in dogs and 0.024 mg/kg orally in cats), they would not be indicated for the prevention

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of metastrongyloid lungworm infection. Similarly, topical selamectin products available in the form of Revolution, utilize average doses of 6 to 6.5 mg/kg selamectin, which results in maximum serum concentrations (C_{max}) in dogs of 12.7 to 22.7 ng/mL,[36] remaining well below the drug concentration necessary to target lungworm larvae examined in this study. Yet, further studies may be warranted in cats, based on a disproportionately higher reported C_{max} of 5,513 ± 2,173 ng/mL after topical dosing with 24 mg/kg selamectin in this species.[25] Monthly heartworm preventive containing moxidectin (Advantage Multi: 2.5 mg/kg for dogs, 1 mg/kg for cats) are also available as topical solutions, for which specific pharmacokinetic data for dogs remains unpublished in the peer-reviewed literature. A study using Advantage Multi in cats (1 mg/kg) showed mean trough levels of moxidectin in blood samples collected before repeated dosing at 28-day intervals of 16.5, 33.4, and 40 ng/mL, respectively.[37] Similarly, a recent study using a different (noncommercial) topical combination product containing moxidectin, demonstrated C_{max} concentrations of 25.3 ng/mL in dogs (6.75 mg/kg topical dose) and 13.6 ng/mL in cats (4.32 mg/kg topical dose), respectively.[38] The latter concentrations in both studies would only be expected to target infective L_3 larvae of *C vulpis*, but not *Ae abstrusus* in cats or *An vasorum* in dogs. The monthly administration of some commercially available products containing milbemycin oxime for dogs (0.5 mg/kg oral Interceptor) may target infective larval stages of *C vulpis* but not *An vasorum* based on the in vitro results.

Although the present study indicates that the tested anthelmintics would not be effective against L_3 of *An vasorum*, other in vivo trials show various drug efficacies against *An vasorum* infection. For instance, a study of dogs experimentally infected with *An vasorum* L_3 and treated with spot-on topical moxidectin (2.5 mg/kg) and imidacloprid (10 mg/kg [insecticide], Advocate) at day 4 and 32 postinfection, demonstrated that the drug had efficacy against the adult stage.[39] Another study demonstrated the persistent effectiveness for 1 month of a topical spot-on containing imidacloprid and moxidectin (Advocate), which was given to dogs 4 weeks before infection with *An vasorum* L_3.[17] A recent experimental study also revealed that oral treatment with 24 mg/kg moxidectin, 1.2 mg/kg sarolaner (ectoparasiticide), and 5 mg/kg pyrantel (Simparica Trio) at 28 days postinfection provided ≥ 92.9% efficacy against recently moulted young adults and mature adult *An vasorum*.[16] Similarly, experimentally infected dogs were treated orally with a combination of milbemycin oxime (0.75 to 1.0 mg/kg) and spinosad (insecticide, 45 to 60 mg/kg) at 30 days postinfection, which provided ≥ 98.8% efficacy.[6] However, after necropsy examinations at day 56 and 58 posttreatment, adult stage of *An vasorum* were detected in 3 out of 8 dogs. In another efficacy study, 4 treatments with a combination of 0.5 mg/kg milbemycin oxime and 2.5 mg/kg afoxolaner (insecticide, NexGard Spectra) at monthly intervals provided ≥ 94.9% efficacy but could not completely prevent the development to the adult stage of *An vasorum*, as adult worm burdens ranging between 1 to 24 worms were recovered in 9 out of 10 dogs.[40] In the latter, it is likely that L_4 survived after the dogs were treated 7 days after exposure. Based on the parasite's life cycle, when the treatment is administered between days 28 to 32 postinfection, the anthelmintics target the young adult and adult stage. Topical moxidectin is the only anthelmintic that can be administered 4 weeks before infection since moxidectin has persistent activity in the body tissues for at least 28 days, and 4 consecutive monthly treatments will lead to a steady-state level.[41] Since topical moxidectin has persistent activity, L_4 could have survived and developed to the young adults and then the drug killed them. On the other hand, milbemycin oxime reached C_{max} in serum after oral administration between 3 to 6 hours and the half-life was about 57 hours.[42] Therefore, these anthelmintics would likely have activity against L_4, L_5, and adult worms, and none of them probably have activity against L_3 based on the in vitro findings.

Currently, macrocyclic lactones including ivermectin, milbemycin oxime, moxidectin, and selamectin are given once a month (oral or topical administration) or by injection of moxidectin every 6 months to kill third and fourth-stage heartworm larvae (L_3 to L_4) that are less than 30 days old.[43] Such treatments are "preventive" in the sense that they limit the duration of infection to a period of time insufficient to result in significant tissue damage. The long development period (6 to 9 months), a long prodromal period (3 to 5 years), and the availability of anthelmintics with efficacy against both the third and fourth-stage larvae are conducive to this type of approach in the protection of dogs and cats from this dangerous pathogen. A similar approach involving the treatment of pets exposed to metastrongyloids before the occurrence of severe organ damage should be superior to administering therapy only after the onset of clinical disease. There are considerable differences in developmental times between *D. immitis* and the species of metastrongyloids. The monthly administration of effective anthelmintics prevents the development of *D. immitis* beyond the L_4 stage from any possible exposure the dog or cat has received in the previous 30 days.[43] In contrast, the final molt to the adult stage occurs by 7 to 9 days post infection in *Ae abstrusus*, *An vasorum*, and *C vulpis*.[44–46] Therefore, monthly use of anthelmintics that lacked efficacy against L_3 or L_4 would require at least one additional treatment at least 1 week after the last potential exposure. At the present time, in vitro testing of anthelmintic efficacy against the L_4 is not possible since no methods exist to culture L_4 in vitro. Further advances in the culture of parasitic nematodes are required before in vitro testing of anthelmintic efficacy against L_4 can be conducted.

In this study, anthelmintic efficacy against 3 species of infective lungworm larvae was based on the inhibition of larval motility in vitro. Previously established in vitro assays used for the detection of

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anthelmintic resistance are commonly based on the effects on the development, movement, and growth of various nematode species from ruminants, dogs, rodents, and humans. These assays include the egg hatch assay, larval development assay, larval migration inhibition assay, larval motility assay, and larval feeding inhibition assay.[9–15] In the current study, our in vitro larval motility assay relied on the addition of a warm digest solution to stimulate larval movement after incubation with various anthelmintics, while other studies determined larval viability after the addition of hot water at 50 °C.[11,12] Alternatively, propidium iodide has been used to indicate larval mortality rate of *Angiostrongylus cantonensis*[47] and *Caenorhabditis elegans*,[15] in lieu of a motility assessment. It is important to emphasize that in the absence of a digest solution, most of the larvae, including those in the control wells, would have been misidentified as "dead or nonviable." Warmed digest solution containing pepsin and HCl was, therefore, used to effectively trigger the larval activity of *C vulpis*, *An vasorum*, and *Ae abstrusus*. Using a digest solution to stimulate larval motility was previously described.[20]

Anthelmintic efficacy in this study was dependent on the lungworm species, drug type, and drug concentration. Lungworms are considered susceptible to a variety of anthelmintics. For example, fenbendazole (25 to 50 mg/kg for 3 to 21 days) has been used based on a wide safety margin and a low probability of side effects.[48,49] A single dose of milbemycin oxime (0.5 mg/kg) or once weekly administration for 4 weeks has also been used effectively against *C vulpis* and *An vasorum*, respectively.[50] Other anthelmintics, such as levamisole have greater toxicity potential, including risks for gastro-intestinal disturbances, neurotoxicity, and immune-mediated conditions. Similarly, ivermectin carries a high risk for neurotoxicity in dog breeds with the ABCB1 mutation.[48] Currently, there are commercially available combinations of moxidectin and imidacloprid (insecticide, Advocate or Advantage Multi) or milbemycin oxime and praziquantel (anticestocide, Milbemax) that have demonstrated efficacy against *An vasorum* and *C vulpis* infection.[2,5,17,49] Additionally, a combination of moxidectin and imidacloprid (insecticide, Advocate), or emodepside and praziquantel (anticestocide, Profender) has been effectively used against *Ae abstrusus* to decrease larval shedding and resolve respiratory disease clinical signs.[3,4] However, the efficacy of these drugs varies based on the dosage and frequency of usage. The in vitro anthelmintic response to antiparasitic agents may, therefore, guide and improve therapy, disease prevention, and clinical outcome in veterinary patients. In summary, eprinomectin, ivermectin, milbemycin oxime, moxidectin, and selamectin have larvicidal effects as assessed by larval motility on *C vulpis* L_3, whereas moxidectin and selamectin were the most active drugs against *Ae abstrusus* L_3. However, *An vasorum* L_3 was less susceptible to any of the anthelmintics tested except for selamectin at high drug concentrations. Use of these anthelmintics prophylactically to effectively treat larvae and prevent the development to the adult stage needs further research.

Ethical statement

The study was approval by the UPEI Animal Care Committee and was conducted in accordance with the Guide to the Care and Use of Experimental Animals available at: ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf.

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