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Citation	Collymore, C., Watral, V., White, J. R., Colvin, M. E., Rasmussen, S., Tolwani, R. J., & Kent, M. L. (2014). Tolerance and Efficacy of Emamectin Benzoate and Ivermectin for the Treatment of Pseudocapillaria tomentosa in Laboratory Zebrafish (Danio rerio). Zebrafish, 11(5), 490-497. doi:10.1089/zeb.2014.1021
DOI	10.1089/zeb.2014.1021
Publisher	Mary Ann Liebert, Inc.
Version	Version of Record
Terms of Use	http://cdss.library.oregonstate.edu/sa-termsofuse

ZEBRAFISH
Volume 11, Number 5, 2014

DOI: 10.1089/zeb.2014.1021

Tolerance and Efficacy of Emamectin Benzoate and Ivermectin for the Treatment of *Pseudocapillaria tomentosa* in Laboratory Zebrafish (*Danio rerio*)

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Abstract

Tolerance of adult zebrafish and efficacy of emamectin benzoate and ivermectin in eliminating *Pseudocapillaria tomentosa* infection were evaluated. In the tolerance study, behavioral changes, fecundity, histopathology, and mortality were evaluated for in-feed administration of emamectin (0.05, 0.10, and 0.25 mg/kg) and ivermectin (0.05 and 0.10 mg/kg). All doses of emamectin were well tolerated. Ivermectin 0.05 mg/kg administration resulted in mild behavioral changes and a transient decrease in fecundity. Ivermectin 0.10 mg/kg administration resulted in severe behavioral changes and some mortality. In the efficacy study, emamectin (0.05 and 0.25 mg/kg) and ivermectin (0.05 mg/kg) were evaluated for their efficacy in eliminating *P. tomentosa* infection. Emamectin reduced parasite burden in infected zebrafish, and ivermectin eliminated intestinal nematode infections. Despite a small margin of safety, ivermectin 0.05 mg/kg was effective at eliminating *P. tomentosa* infection in adult zebrafish. Higher doses or a longer course of treatment may be needed for complete elimination of *P. tomentosa* infection using emamectin. In this study, we propose two possible treatments for intestinal nematode infections in zebrafish.

Introduction

Pseudocapillaria tomentosa is a nematode pathogen of the proximal intestinal tract in zebrafish.[1] Transmission occurs through the ingestion of infective eggs[1,2] or infected paratenic hosts (e.g., *Tubifex* spp. worms).[2] Infected fish are often asymptomatic; however, emaciation and skin darkening may also be evident.[3] On histopathological examination, cross sections of nematodes embedded in the mucosal epithelium may be observed as well as severe inflammatory changes in the intestine and coelomic cavity.[3] Characteristic double-operculated barrel-shaped eggs may also be visualized in the gut lumen. Chronically infected zebrafish may present with severe atrophy of the proximal intestinal mucosa, but few to no parasites.[3] Reported effective treatments for this parasite in zebrafish are sparse.[4,5]

In production fisheries, medications such as avermectins and benzimidazoles are used to treat parasitic infections.[6,7] Avermectins act on nervous system ligand-gated chloride channels to increase the permeability of cell membranes to chloride, leading to dysfunction and death of parasites.[8,9] Emamectin benzoate was originally developed as an insecticide for pest control of plant crops.[6,7,9] In aquaculture, emamectin premix (SLICE 0.2%; Merck Animal Health, Summit, NJ) top-coated onto feed is extensively used to treat ectoparasitic crustacean infestations in trout and salmon.[6,7] It is characterized by a wide margin of safety, high efficacy, and long residual action.[6] Ivermectin is used to treat parasites in both aquatic and mammalian species.[7,8,10] It is effective for treating parasitic infestations through top-coating onto feed, but is not extensively used in aquaculture due to its narrow margin of safety.[6,11]

This study had two goals. The first was to evaluate the safety and effects on fecundity of emamectin and ivermectin in adult zebrafish. We hypothesized that emamectin at all doses would not result in morbidity and mortality or decreased fecundity. We also hypothesized that at low doses ivermectin would not result in morbidity, mortality, and decreased reproductive output. The second goal was to assess

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the efficacy of each drug in eliminating *P. tomentosa* from infected zebrafish. We hypothesized that both drugs would effectively eliminate infections.

Materials and Methods

Tolerance and fecundity studies were conducted at the Rockefeller University (RU), and efficacy studies were conducted at the Oregon State University (OSU). Hence, we described methods for both institutions separately.

Tolerance and fecundity animals and housing

Animals in the tolerance and fecundity study were housed in an AAALAC International-accredited facility in compliance with the *Guide for the Care and Use of Laboratory Animals (The Guide)*.[12] All research procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the RU. Adult AB wild type line zebrafish ($n = 140$; male and female; age, 4 months), originally obtained from Carolina Biologicals (Burlington, NC) and bred for four generations at the RU, were used for this study. Fish were considered free of *Pseudoloma neurophilia*, pathogenic *Mycobacterium* spp. (*M. marinum* and *M. haemophilum*), *P. tomentosa*, and *Edwardsiella ictaluri* based on twice yearly gross and histopathologic examination of no less than five colony fish per rack, and PCR testing for *P. neurophilia* and *Mycobacterium* spp.

Animals were housed at a density of 10 mixed-sex fish/2.5-L tank on a recirculating housing system (Marine Biotech, Apopka, FL) with mechanical and biological filtration as well as ultraviolet disinfection, using carbon-filtered municipal tap water balanced to pH 7.0–7.5 with sodium bicarbonate (catalog no. S233–500, Sodium Bicarbonate, certified ACS; Fisher Scientific, Fairlawn, NJ) and a conductivity of 500–600 μS maintained with a marine salt mixture (Instant Ocean®; United Pet Group, Inc., Cincinnati, OH) in a room with a 14:10 light:dark photoperiod with lights on at 900 at 28°C. Fish were fed a mixture of a commercial pelleted diet (Adult Zebrafish Complete Diet; Zeigler Brothers, Gardners, PA) and an artificial *Artemia nauplii* replacement diet (Golden Pearls Reef and Larval Fish Diet 300 to 500 μm; Brine Shrimp Direct, Ogden, UT) twice daily.

Tolerance and fecundity study experimental design

Experimental groups ($n = 20$/group) were as follows: untreated (no anthelminthic), emamectin (0.05, 0.10, and 0.25 mg/kg), and ivermectin (0.05 and 0.10 mg/kg). One tank of 10 fish per group was composed of 5 male and female pairs to evaluate reproductive outcomes for 2 weeks prior and 8 weeks following start of treatment. The second tank of 10 fish per group was used to determine if lesions due to drug toxicity were present on histopathological evaluation. Emamectin groups were treated with medicated feed twice daily for 7 consecutive days for a total of 14 treatments. Ivermectin groups were treated twice daily twice a week for 4 weeks for a total of 16 treatments. After each feeding, fish were observed for behavioral changes and mortality. On the day after final treatment (day 8 for emamectin groups and day 29 for ivermectin groups), 10 fish per group were submitted for whole-body histopathological evaluation.

Medicated feeds preparation and administration

Medicated feeds for this portion of the study were prepared 1 week before treatment based on an approximate weight of 0.6 g/fish and a feeding rate of 5% body weight divided between both feedings daily. Both emamectin premix powder (SLICE® 0.2%; Merck Animal Health) and ivermectin powder (99% analytical grade; Sigma Aldrich, St. Louis, MO) were added and mixed into 200 g of the commercial pelleted diet (Adult Zebrafish Complete Diet; Zeigler Brothers) using a wooden tongue depressor. Analytical grade ivermectin was chosen and approved by both IACUCs as pharmaceutical grade preparations contain vehicles with unknown effects on zebrafish. The feed was then spread over wax paper and sprayed with vegetable oil (Crisco vegetable oil; The J.M. Smucker Company, Orrville, OH), heated over a stove until mild boiling was observed, and calculated at 0.5% w/v of feed weight to adhere the medication to feed pellets. The feed air-dried overnight before being placed into plastic sealable containers (Ziploc® brand containers with the Smart Snap® seal, Ziploc; S.C. Johnson and Son, Inc., Racine, WI). The control diet did not contain any medication, but was coated with oil to minimize variation between feeds.[9] Diet was maintained at 4°C until the time of administration to the fish.

The designated feed was administered to each tank at 9:30 and 16:00 hours on treatment days. *A. nauplii* was withheld during the entire treatment period. Water flow to each tank was halted for 1 h immediately before feeding, preventing feed from exiting the tank before ingestion. After 1 h, excess feed was siphoned from the tank bottoms and water flow restored. Approximate quantity eaten by the fish was visually assessed by observing the fish consuming the feed and observing the quantity of feed siphoned from the tank bottoms. Carbon filters on the recirculating life support system prevented excess drug from potentially reentering the system and confounding the results.

Medicated feed concentration determination

A 60 g sample of each feed after preparation, as described above, was submitted to reference laboratories for drug concentration analysis. Both emamectin and ivermectin were measured according to previously published methods.[13,14] Emamectin was analyzed by HPLC analysis by Eurofins Lancaster Laboratories, Inc. (Lancaster, PA).[13] Ivermectin was analyzed by HPLC analysis by the Memorial Sloan Kettering Cancer Center Analytical Pharmacology Core Facility (New York, NY).[14]

Observations

Fish were observed for clinical signs of toxicity during each 1-h feeding period, and then 30 min, 1 h, and 2 h after feeding for 5 min. Observations included scale color changes, aberrant swimming behavior, lethargy, and mortality.[6,10,15] If fish displayed severe changes such as paralysis or flared opercula, they were immediately euthanized.

Fecundity

Two weeks before the treatment, five pairs of fish from each group were bred weekly to obtain baseline reproductive data. For the week of emamectin treatment and the first week

of ivermectin treatment, no fish were bred. To induce spawning, male and female fish were placed on opposite sides of clear divided breeding tanks with false bottoms (Aquatic Habitats, Apopka, FL) 15 h before the next photoperiod. Fifteen minutes after the light period onset, the divider was removed and each pair was allowed to spawn for 3 h, after which the fish were returned to their home tank. Each group was bred weekly for 8 consecutive weeks to evaluate effects on fecundity. Fecundity was evaluated by counting the number of fertilized eggs laid immediately following the 3-h spawning period.

Necropsy and histopathology

Fish were euthanized using rapid chilling according to the *AVMA Guidelines for the Euthanasia of Animals*: *2013 Ed.* and examined for gross lesions. The ventral coelom was incised and the fish placed into the Bouin's solution for a minimum of 24 h. To prepare histopathologic slides, fish were removed from the Bouin's solution and sectioned with a sharp blade parasagittally. Both the longitudinal sections were processed using standard methods and embedded in paraffin. Two 4-μm-thick sections were taken at 100 μm levels and stained with hematoxylin and eosin, for evaluation by a board-certified veterinary pathologist.

Efficacy study

Fish in the efficacy study were housed in an AAALAC International-accredited facility in compliance with *the Guide*.[12] All procedures were approved by the IACUC at OSU. Adult, mixed-sex, wild-type 5D line zebrafish ($n = 200$) from the Sinnhuber Aquatic Research Laboratory were experimentally infected by placing them into a 2-ft-diameter (133 L) circular tank that had previously housed $\sim$100 *P. tomentosa* infected Casper zebrafish for 1 month. 5D fish were considered free of *P. neurophilia*, pathogenic *Mycobacterium* spp. (*M. marinum* and *M. haemophilum*), *P. tomentosa*, and *E. ictaluri* based on quarterly sentinel histopathology and PCR testing for *P. neurophilia* and *Mycobacterium* spp. Casper fish had an unknown health history. After a 7-day exposure, fish were removed and housed in mix-sexed groups of 15 fish/tank in 2.8-L tanks on a flow-through system (Aquaneering, San Diego, CA) with pH 7.0 and an average conductivity of 125 μS maintained in a room with a 14:10 light:dark photoperiod with lights on at 07:00 at 28°C.

Experimental groups, each comprised three tanks and containing a total of approximately 45 fish, included treated nematode exposed (positive controls); untreated unexposed (negative controls); nematode exposed treated with emamectin at 0.05 and 0.25 mg/kg; and nematode exposed treated with ivermectin 0.05 mg/kg. The emamectin diet was fed once a day for 7 days and ivermectin diet fed twice a week, on Monday and Thursday, for a 4-week total of eight feedings. Fish were fed either medicated or untreated diets once daily at 8:00 am. The base diet was the same as used at the RU, except that fish were not supplemented with *A. nauplii*. Total feed was targeted at 3% body weight/day. This target was chosen as fish in the tolerance study were observed eating only half the feed provided at each feeding. To reduce feed waste and ensure all feed was consumed, the target concentration was reduced and provided only once daily.

Three and 4 weeks after the initiation of emamectin and ivermectin diet administration, respectively, 4–8 fish/tank (approximately half the fish in each tank) were euthanized with 500 mg/L buffered MS-222 (Argent Chemical Laboratories, Redmond, WA). The entire gastrointestinal tract was removed and wet mount preparations examined at 100× and 200× to determine adult and larval worm burden and female worm maturity. Female worm maturity was determined by observing the presence of developing eggs in its reproductive tract.

Medicated feed preparation and administration

New medicated feeds were prepared 1 month before the treatment, as described above, based on the estimated average weight for all fish per tank and a 3% body weight feeding rate. Estimated fish weights were obtained to administer accurate dosages. Individual fish were removed from their tank placed in a beaker of water on a tared scale. Diets were maintained at 4°C beginning after the drying period ended until day of administration to the fish. Actual weights of individual fish were obtained at intestinal sampling times by gently drying the fish with a paper towel and placing them on a scale.

Statistical analysis

Statistical analysis was conducted to evaluate the effect of treatments on zebrafish fecundity, total worm burden, mature worm burden, parasite incidence, cumulative mortality, and weight data. Parasite counts are commonly assumed to follow a negative binomial distribution.[16] However, in this study, all ivermectin-treated fish cleared infection (i.e., tanks where worm counts = 0) at 5 weeks, violating the negative binomial constraint that mean worm counts must be greater than zero. Therefore, total and mature worm counts were averaged for each tank and used in analyses. Geometric and arithmetic means were calculated and presented. Similarly, parasite incidence and cumulative mortality were summarized for each tank for analysis.

Linear and generalized linear models, including effect of drug treatment, week, and their interaction, were fit to fecundity, mean total worm, mean mature worm, and individual weight data. The model fit to individual fish weight data also included an effect of the tank. Generalized linear models assuming negative binomial distributions were fit to tank incidence (number of infected hosts/sample size) and cumulative mortality data (number of mortalities/number in tank). These models differ from linear models by incorporating the sample size when evaluating proportions (i.e., incidence, mortality).

For each response, a full model was fit to the data that included the main treatment and week effects, as well as their interaction. The interaction of treatment and week was insignificant and dropped from the model for all analyses. The final models for each analysis included main effects of treatment and week, with the exception of fecundity, which retained only the effect of treatment. Model assumptions were visually assessed for the fitted models and found to be adequate for statistical inference. An analysis of variance (ANOVA) was performed for the final fitted models to evaluate the effect of treatment and week. Statistical significance was set at $\alpha = 0.05$ for evaluating model factors.

Table 1. Expected and Actual Concentrations of Emamectin and Ivermectin in Medicated Feeds of the Tolerance Study ($N = 1$/Group)

Medication	*Expected concentration (mg/kg)*	*Actual concentration (mg/kg)*	*Difference in concentrations*
Untreated	0	< 0.01	0
Emamectin	0.05	0.63	12.6×
	0.10	1.13	11.3×
	0.25	2.56	10.24×
Ivermectin	0.05	0.25	5.15×
	0.1	0.45	4.49×

Comparisons among treatments controlling for the effect of week in models, including this term, were evaluated using Tukey's pairwise comparisons.[17] A Bonferroni correction was made to maintain an overall $\alpha = 0.05$ by dividing α by the number of comparisons made.[18]

Results

Toxicity and fecundity experiments

Medicated feed concentrations. Feed analysis revealed a higher than expected concentration for both experimental diets. The concentrations were 10 and 5 times higher than expected for the emamectin and ivermectin diets, respectively (Table 1).

Behavioral observations. No behavioral changes or mortality was observed in any fish treated with emamectin. Fish treated with ivermectin, at 0.05 mg/kg, displayed a darkened body color, reduced movement, and incoordination within 30 min after initiating treatment. Four of the 20 fish treated, with ivermectin at 0.1 mg/kg of ivermectin, displayed cessation of movement and erratic swimming behavior as shown by periods of immobility followed by body twitching and an inability to swim upright within 30 min of initiating treatment; the fish also appeared to be in respiratory distress as evidenced by markedly flared opercula and rapid opercular movements. Given the severity of clinical signs observed, these four fish were immediately euthanized and submitted for necropsy. The remaining fish also showed similar, but less severe clinical signs 1–2 h after initiating treatment. All remaining fish survived until the end of the experiment.

Fecundity. The total number of fertilized eggs counted per week per group is provided in Table 2. There were no statistical differences between controls and treated fish.

Necropsy and histopathology. No lesions were observed in any fish.

Efficacy experiment

Drug delivery. Feed concentration in this portion of the study was not determined. Initial drug concentrations were based on an initial estimated mean of 0.35 g wet weight per fish. Comparison of dry and wet weights at 4 week posttreatment (Table 3) showed that wet weights were between 11% and 15% greater. Therefore, the actual dose fed to the fish was slightly higher than predicted.

Nematode abundance and incidence. Approximately half the fish from each of the three tanks for each treatment group were examined at 4 and 5 week posttreatment and similar results observed within each group (Table 3). Fish from all treatment groups exhibited lower incidence and worm burden as compared to positive controls (Table 3). Only 10% of the fish treated with high-dose emamectin were infected at 5 weeks and their worm burdens were less than 10% of the worm burdens observed in untreated nematode-exposed fish. In addition, in the latter group, many gravid female nematodes were observed. The limited number of nematodes observed in the treated fish was mostly immature. Only three mature worms were observed in the low-dose emamectin group. Three dead encapsulated worms were observed in three different fish from the high-dose emamectin group. These were not included in the overall analysis as they were not viable.

ANOVA using the Bonferroni correction indicated that the high-dose emamectin and ivermectin groups had significantly fewer worms compared to positive controls (Tukey's pairwise comparison; $p < 0.008$). The efficacy of low-dose emamectin was significant without using the Bonferroni correction (ANOVA; $p < 0.05$). High-dose emamectin and ivermectin had similar nematode burdens. High-dose emamectin had a significantly lower burden than low-dose emamectin (Tukey's pairwise comparison; $p < 0.008$). Ivermectin also had a statistically significant lower burden without the Bonferroni correction (ANOVA; $p < 0.05$).

Interestingly, the pattern of worm burden, particularly in treated groups, fits best with a negative binomial distribution

Table 2. Total Number of Fertilized Eggs per Group Per Week ($N = 5$ Pairs/Week) Before and After Treatment With Emamectin and Ivermectin

		Week										
Medication	*Dose (mg/kg)*	*−2*	*−1*	*Tx*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Untreated	0	1381	1294	N/A	1184	1125	1197	1142	1252	1208	974	1380
Emamectin benzoate	0.05	1032	1650		1967	1468	1178	1125	1150	1291	1181	1291
	0.10	1638	996		2026	1080	N/B	1897	1129	751	1080	1058
	0.25	648	334		1765	1388	1474	1522	1525	1465	1115	1750
Ivermectin	0.05	812	494		0	0	0	976	814	1075	1317	1000
	0.1	1432	571		610	0	487	658	812	1712	1375	900

TABLE 3. ABUNDANCE, INCIDENCE, CUMULATIVE MORTALITY, AND WEIGHT IN ZEBRAFISH INFECTED WITH *PSEUDOCAPILLARIA TOMENTOSA* AND SUBJECTED TO VARIOUS TREATMENTS

	Untreated (positive control)		*Emamectin 0.25 mg/kg*		*Emamectin 0.05 mg/kg*		*Ivermectin 0.05 mg/kg*		*Unexposed (negative control)*	
	4 weeks	*5 weeks*	*4 weeks*	*5 weeks*	*4 weeks*	*5 weeks*	*4 weeks*	*5 weeks*	*4 weeks*	*5 weeks*
Total worms Avg. ± SD (range)	7.8 ± 5.0 (2–18)	4.5 ± 1.8 (1–7)	0.70 ± 1.7* (0–7)	0.33 ± 0.90* (0–3)	4.6 ± 6.0* (0–19)	1.3 ± 1.7* (0–6)	2.4 ± 5.4* (0–21)	0*	0	0
Mature worms Avg. ± SD (range)	1.0 ± 1.5 (0–5)	1.2 ± 1.3 (0–4)	0*	0*	0.11 ± 0.46* (0–2)	0.22 ± 0.73* (0–3)	0.063 ± 0.25* (0–1)	0*	0	0
Avg. incidence (%)	19/19 (100)	17/17 (100)	4/20* (20)	2/20* (10)	2/19 (11)	0/19 (0)	6/16* (38)	0/19* (0)	0/15 (0)	0/15 (0)
Cumulative mortality (%)	4/38 (11)	6/38 (16)	0/38 (0)	1/38 (3)	4/39 (10)	4/29 (14)	9/38 (24)	9/38 (24)	0/15	0/15
Weight (g) Avg. ± SD	0.23 ± 0.079	0.22 ± 0.068	0.29 ± 0.074*	0.28 ± 0.051*	0.28 ± 0.047*	0.27 ± 0.064*	0.24 ± 0.077	0.29 ± 0.041	0.51 ± 0.13	0.41 ± 0.049

Treated zebrafish examined at 4 and 5 week postexposure.
*Treatment was statistically different from positive control using Bonferroni correction ($\alpha = 0.008$). Negative controls were not included in the analyses.

(Fig. 1). Infections in treated fish were aggregated in a few fish, with most demonstrating few to no nematodes. For the positive control group, this pattern was maintained; however, all fish were infected with at least one worm.

Regarding incidence, all untreated nematode-infected fish were infected at both time points examined. In comparison, the maximum incidence (38%) in the ivermectin group was observed at 4 weeks posttreatment (Table 3). The incidence of infection was statistically higher in the positive control groups at both 4 and 5 weeks posttreatment than high-dose emamectin and ivermectin, but not low-dose emamectin. Differences between high-dose emamectin and ivermectin were not significant.

Mortality. No negative control fish and only one fish from the high-dose emamectin group died during the experiment (Table 3). The positive control and ivermectin-treated groups had the highest mortality; 16% and 24%, respectively. Low mortality in the high-dose emamectin group trended toward significance (Tukey's pairwise comparison; $p = 0.092$) compared with the positive control group.

Terminal fish weights. Individual weights obtained at 4 and 5 weeks posttreatment were variable within groups (Table 3). For example, gravid fish in the negative control group weighed as much as 0.676 g, whereas the smallest males weighed 0.336 g. At both 4 and 5 weeks posttreatment, the positive control fish weighed less than all the treated groups. Both emamectin groups were significantly heavier than positive control fish (Tukey's pairwise comparison; $p < 0.008$). Fish in the ivermectin group were only marginally heavier than the positive controls (Tukey's pairwise comparison; $p = 0.0502$). Negative control fish were $\sim$1.5 to 2 times heavier than fish exposed to the parasite, regardless of the treatment regimen.

Discussion

Emamectin

In the tolerance and fecundity study, no differences in behavioral observations, fecundity, or mortality were observed in zebrafish treated with emamectin, suggesting this drug is well tolerated by zebrafish. Previous reports have found emamectin to be safe even at 10 times the recommended dose in salmonids.[6,7] Given the miscalculation that occurred in feed preparation, this also appears to be true for zebrafish. In other fish, no effects on fecundity of treated adults have been reported.[6,7,9] We also observed no effects on fecundity in our study. To the author's knowledge, effects on zebrafish embryos exposed to emamectin have not been previously reported.

No specific histopathological lesions were attributed to drug treatment in the zebrafish, similar to previous reports in other species.[6,7] Emamectin has several advantages for the treatment of nematode infections in zebrafish. It has a wide margin of safety, is easily incorporated into a diet formulation, and does not affect fecundity. It is reported to have a long residual effect (6–9 weeks) in salmonids due to accumulation in tissues, providing potential protection for a considerable time after treatment is withdrawn.[6]

Emamectin is usually used to control crop nematodes and arthropod pests or parasites.[6,7,9] Efficacy against ectoparasitic

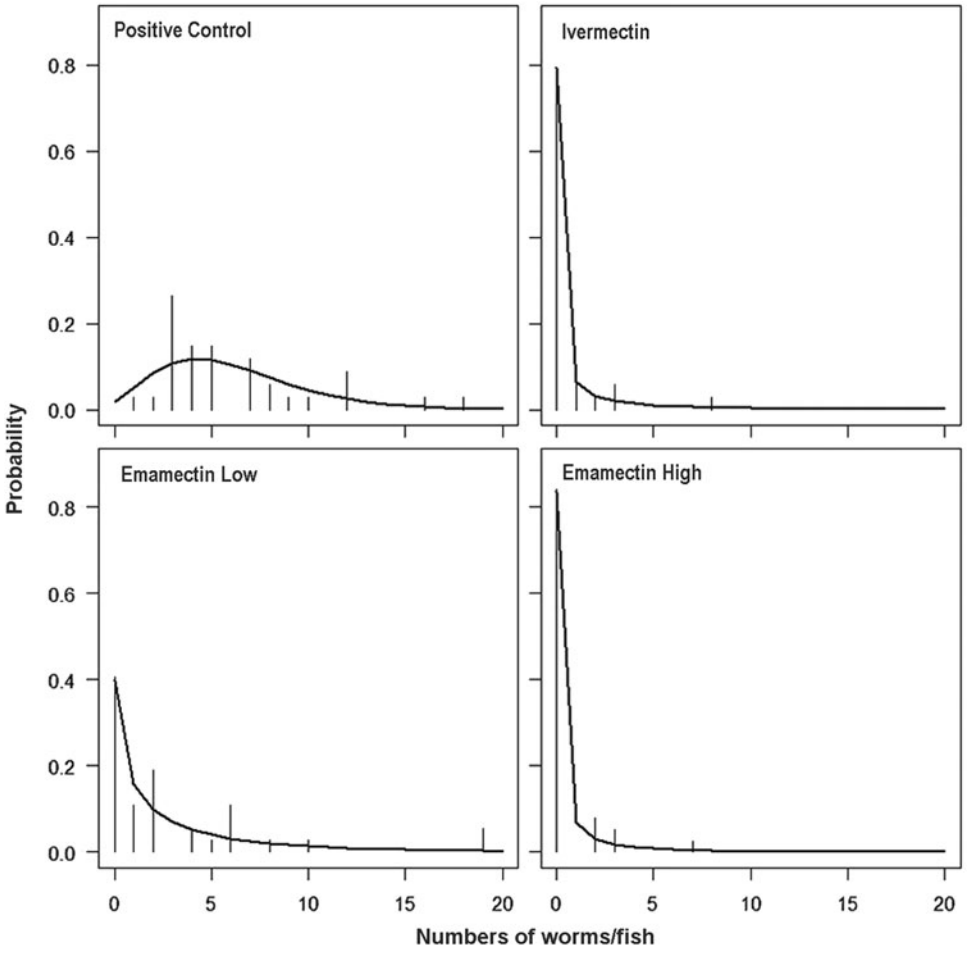

FIG. 1. Negative binomial distribution of *Pseudocapillaria tomentosa* fit to parasite counts in treated and positive control zebrafish by maximum likelihood. Estimated mean (μ) and dispersion (k) parameters of fitted negative binomial distribution (*solid lines*) were Positive control, $\mu = 6.1, k = 4.77$; Ivermectin, $\mu = 1.47$ and $k = 0.086$; Emamectin low dose, $\mu = 3.00$ and $k = 0.45$; Emamectin high, $\mu = 0.50$ and $k = 0.099$. Y-axis, probability (0–1).

infestations in salmonids and goldfish was demonstrated.[6,9] Only one previous report has demonstrated limited efficacy against intestinal nematodes.[19] The authors found that one dose of emamectin benzoate gavaged to the American eel (*Anguilla rostrata*) resulted in the death of 40% of the adults of *Anguillicoloides crassus*, a swim bladder nematode.[19] In our efficacy study, 0.25 mg/kg was highly effective in reducing but not completely eliminating nematodes. Fish treated with this dose demonstrated no evidence of mature nematodes capable of reproducing and laying more eggs. With a dose of 0.05 mg/kg, a few fish maintained a small number of mature nematodes. This suggests that for zebrafish, either a higher concentration or a longer dosing period is needed to effectively eliminate *P. tomentosa* infection. Overall, the 0.25 mg/kg dose effectively reduced or eliminated nematode infection in most zebrafish.

Ivermectin

In contrast, both doses of ivermectin lead to behavioral changes and decreased fecundity, with moribund fish noted in the higher dose. Ivermectin-treated fish also demonstrated 24% mortality in the efficacy study. We concluded that mortality was due to ivermectin in the latter experiment because treated fish had a higher mortality rate, but fewer nematodes, compared with positive control fish. These findings support previous literature indicating that ivermectin has a poor margin of safety in fish.[8] Ivermectin was shown to efficiently pass through the blood–brain barrier of fish, and it has been hypothesized that ivermectin accumulates in the fish brain because the activity of P-glycoproteins in the blood–brain barrier is substantially lower than in mammals.[6] The P-glycoproteins may be responsible for reducing the ability of ivermectin to enter and accumulate in the brain. Toxicity is thought to be due to the direct action of ivermectin on central nervous system neurotransmitters.[6] In mammals, they can also cause side effects by augmenting the release of the inhibitory neurotransmitter γ-amino-butyric acid (GABA), resulting in increased binding of GABA to postsynaptic nerve terminals in the central nervous system.[8] Previously reported clinical signs in fish associated with ivermectin toxicity included lethargy, incoordination, darkening of scale color, down-rolling of eyes, anorexia, and variable mortality.[6,8,10,11] As in previous reports, we did not find any histopathological lesions related to the behavioral changes observed in fish treated with ivermectin.[6,11] Previous work reported sublethal effects on zebrafish embryos, including malformations, side-laying embryos, tremors, reduced movements, and altered heart rates, after 144 hours postfertilization (hpf) of exposure to ivermectin.[20] Ivermectin has only been documented to persist in fish tissues for up to 28 days.[6] In the efficacy study, despite the side effects of ivermectin, it effectively cleared nematode infections in most fish at 4 weeks posttreatment and all fish at 5 weeks posttreatment. This

suggests it can also be used to eliminate nematode infections in zebrafish. Care must be taken not to over feed the diet as severe morbidity and some mortality may occur.

Pack *et al*. reported clearance of *P. tomentosa* in zebrafish using a combination of trichlorfon and mebendazole (Fluke Tabs; Aquarium Products, Glen Burnie, MD) that is no longer available.[5] Maley *et al.* also reported clearance of the parasite using *A. nauplii* soaked in fenbendazole at a dose of 0.125 mg/L daily for 3 days with repeated treatment in 2 weeks.[4] While both reported effective treatment of nematode infection, no controls were used in either study. High rates of mortality in the embryos of fenbendazole-treated fish were also reported.[4] The no observable effect concentration for zebrafish embryos for fenbendazole was found to be 0.02 mg/L when exposed for 24 hpf.[20] Embryos laid after fenbendazole treatment was completed were normal.

Medicated feed administration

Top-coating medication onto feed is a cost-effective method for oral administration of specific drugs for special needs in production aquaculture or research.[6] Despite this, there are many drawbacks to this method, some of which extend to oral treatments of large populations of fish in general. These issues induced some variation in drug delivery in our study. First, given the small amount of food needed to treat zebrafish, there can be inaccuracies in the preparation of medicated diets due to hand-mixing small amounts of drug into feed.[10] Second, oral dosing can result in uneven exposure of fish due to differences in feeding. Similarly, given that zebrafish can vary in size (gravid females vs. males, for example), accurate dosing is difficult to calculate for all fish without overdosing some fish and underdosing others. Finally, it is difficult to determine how much drug leached out of the feed into water, reducing its efficacy.[11] We closely monitored feeding in the efficacy study. Inevitably, despite our efforts to ensure complete feed consumption, some food was uneaten, which may have led to suboptimal levels of dosing.[11] Therefore, it is difficult to determine the final dose per fish, which may help perpetuate infection due to inaccurate dosing. Perhaps, the few fish with persistent infections in the treatment groups received an inadequate amount of drug. Regardless of all these drawbacks, a significant and dramatic reduction in nematode burdens at both time points was observed in all treated experimental groups.

Parasite distribution

A well-recognized paradigm in parasitology is that macroparasites, such as nematodes, in wild animals generally exhibit a negative binomial distribution, with relatively few hosts harboring most parasites.[16,21] This type of distribution extends to fish as well as domestic terrestrial hosts.[22,23] It is remarkable that this distribution was seen here with a controlled laboratory study, in which all fish were exposed equally to the nematodes. One explanation for this distribution for the treated groups is that heavy infections occurred in fish that consumed less medicated diets, as discussed above. Untreated fish also had a similar pattern of infection. Another factor, which may contribute to infection burden variability, is the cortisol level of fish in the study. Zebrafish normally exhibit a wide variance in the cortisol level, and this might provide one explanation for the variability seen in infections under these controlled conditions.[24] Fish with higher levels of cortisol may have been more susceptible to infection or less able to fight infection.

Geometric versus arithmetic mean

We evaluated differences between the groups using arithmetic and geometric means, but data for the latter are not presented here. The geometric mean is often used for efficacy studies, but for parasite data with negative binomial or other skewed distributions, this often underestimates efficacy.[25,26] Regardless, both calculation methods showed significant efficacy, particularly for the high-dose emamectin group.

Infection interactions

Although Casper fish used in this study had an unknown health history and may have been infected with *Mycobacterium* spp. and *P. neurophilia*, both infections require more than 6 weeks of exposure to become clinical. Histopathology was not performed on these fish; however, the likelihood of transmission to the 5D fish, given the short exposure period, is low.

Future work

Future studies with emamectin may include evaluating the efficacy of a higher dose or a longer exposure than 0.25 mg/kg for 7 days. Also, if higher doses are found to be more effective, verifying that fecundity is not affected would be essential. Following the offspring of treated adults to ensure their fecundity is not affected would also be important, as zebrafish are frequently used to produce embryos for scientific study. As zebrafish are held in smaller tanks than other fish typically treated in aquaculture, it is possible that bath or dip treatments of emamectin may also be effective at treating nematode infections. Evaluating the pharmacokinetics and duration of efficacy in zebrafish will help determine a redosing period if needed. And finally, evaluating direct toxicity to embryos would validate results found here. Similarly, evaluating lower doses of ivermectin for efficacy, behavioral changes, fecundity, and mortality could also prove useful. In addition to further efficacy studies with emamectin, we are investigating methods to disinfect facilities (tank systems, etc.) through physical and chemical methods.

Recommendations

In general, we do not recommend treatment of whole colonies for *P. tomentosa* at this time. Treatment may introduce new experimental variables and lead to other changes not accounted for in research work. Treatment may be an option, or even desired, for fish on quarantine systems that have an unknown health history or are shown to be infected by parasites. Alternatively, valuable fish such as new transgenic lines may also need to be treated to preserve the line. Every effort should still be made to use surface disinfected eggs to populate a clean facility. Professional judgment should be used when deciding whether to treat infected colonies.

Acknowledgments

The authors thank Jim Bowker, Aquatic Animal Drug Approval Partnership Program and Dr. Richard Endris, Merck Animal Health for provision of emamectin benzoate.

Various portions of this study were funded by Merck Animal Health, Comparative Bioscience Center of the Rockefeller University and grants from the National Institutes of Health NIH 8R240D010998-11S. The authors also thank Dr. Neil Lipman for critical review of the article.

Disclosure Statement

No competing financial interests exist.

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