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Efficacy of selected oral chemotherapeutants against *Ichthyophthirius multifiliis* (Ciliophora: Ophyroglenidae) infecting rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: The chemotherapeutic efficacy of 6 in-feed compounds against *Ichthyophthirius multifiliis* Fouquet, 1876 was assessed using experimental infections of rainbow trout *Oncorhynchus mykiss* (Walbaum) fingerlings. Trial doses of 104 ppm amprolium hydrochloride or 65 ppm clopidol fed to fish for 10 d prior to infection significantly reduced the number of trophonts establishing in trout fingerlings by 62.0 and 35.2% respectively. In-feed treatments of infected trout with either 63 or 75 ppm amprolium hydrochloride, 92 ppm clopidol, or 38, 43 or 47 ppm salinomycin sodium for 10 d also significantly reduced the number of surviving trophonts by 77.6 and 32.2% for amprolium, 20.1% for clopidol and 80.2, 71.9 and 93.3% respectively for salinomycin sodium.

KEY WORDS: *Ichthyophthirius multifiliis* · Whitespot · Amprolium · Salinomycin sodium · Clopidol · *Oncorhynchus mykiss*

INTRODUCTION

The holotrichous ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 is regarded as one of the most pathogenic diseases of freshwater fishes (Matthews 1994). Infections in farmed fishes are common (Valtonen & Keranen 1981), but epizootics do occasionally occur in wild fishes (e.g. Traxler et al. 1998). Within the UK, the British Trout Association has estimated that losses to the trout industry were in the region of 5% of infected batches with losses of between 30 and 80% in certain cases and with ca. 30% of British trout farms affected by *I. multifiliis*.

Of the chemicals that were once commonly used against ichthyophthiriasis in food fishes, such as the rainbow trout *Oncorhynchus mykiss* (Walbaum), the use of dimetridazole in food-producing animals has been banned by the European Commission because of its considered carcinogenicity to humans (Anonymous 1995), as has malachite green which is also suspected to be mutagenic. At present, only formalin and chloramine-T are permitted for use against *Ichthyophthirius multifiliis* in the European Union, and the activity of these chemicals is limited; they are effective only in killing the external stages of the parasite and multiple bath treatments are necessary for its control. Thus, there is a real need to find an efficacious in-feed treatment to replace dimetridazole and malachite green. For *I. multifiliis*, a parasite situated within the epithelium of the host, an in-feed treatment represents a more appropriate system of drug delivery. Before reaching their target, in-feed compounds are not compromised by environmental conditions (water solubility, activity diminished by organic loading, pH, O_2 etc.) to the same degree as bath treatments; they can have a longer window of activity, are less stressful to administer, and the by-products generated by the host tissues can be more efficacious than the parent compound. In-feed compounds, however, depend upon the host being sufficiently hungry to take the medicated diet. The efficacy of 6 in-feed anticoccidiostats, amprolium hydrochloride, clopidol, decoquinate, monensin, nicar-

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bazin and salinomycin sodium, against *I. multifiliis* were assessed (Table 1).

Although more commonly used as an anti-coccidial in lambs and in cattle, decoquinate has also been successfully used for the treatment of *Hepatozoon americanum* infection in dogs (MacIntire et al. 2001), and has been demonstrated to control ovine toxoplasmosis (Buxton et al. 1996). Amprolium, clopidol, monensin, nicarbazin and salinomycin sodium are all commonly used for the treatment of poultry infected with *Eimeria* spp. (Arakawa et al. 1991, Peeters et al. 1994, Daugschies et al. 1998). Monensin, salinomycin and nicarbazin have been used to completely inhibit the growth of *Cryptosporidium parvum* (Armson et al. 1999), and salinomycin sodium has been demonstrated to reduce the incidence of shedding of *Salmonella enteritidis* in broilers at 6 wk post-infection (Bolder et al. 1999) and to cause irreversible damage to the plasmodial developmental stages of *Henneguya* sp. (Dohle et al. 2002).

MATERIALS AND METHODS

Fish husbandry. To assess the efficacy of the various compounds, *Oncorhynchus mykiss* (Walbaum) with a mean weight of 6.5 g were obtained from a commercial hatchery and were acclimated in 50 l flow-through tanks for 2 wk prior to the start of a treatment. The water supplied to the tanks was carbon-filtered and the temperature within the tanks was maintained at 15°C. When the incoming water temperature dropped to a point at which it was no longer possible to heat tanks on a flow-through system and maintain a steady 15°C, then static tanks were used. The trout were fed a commercial pelleted feed (Trouw Nutra Trout Fry 03) top-dressed with cod liver oil, at 1% body weight d^{-1}. Those fish receiving medicated feed for a pre-determined period prior to theront exposure were identified by means of individual dye marks (1% alcian blue in distilled water) on the ventrum.

***Ichthyophthirius multifiliis* infection protocol.** Tomonts of *Ichthyophthirius multifiliis* derived from brown trout *Salmo trutta* L. were obtained from R. A. Matthews, University of Plymouth, UK, and a culture was maintained in *Oncorhynchus mykiss* held in aquaria at the Institute of Aquaculture, University of Stirling. Mature trophonts were collected with a glass pipette as they exited their fish hosts, or were recovered from the bottom of plastic tanks containing infected stock fish. These tomonts were transferred to pre-rinsed plastic Petri dishes containing 50 ml carbon-filtered, dechlorinated, 15°C water, and were main-

Table 1. Details of chemotherapeutants tested in this study

Compound	Properties
Amprolium hydrochloride	
Synonym:	(1-[(4-amino-2-propyl-5-pyrimidinyl)-methyl]-2-methyl pyridinium chloride hydrochloride
Chemical formula:	$C_{14}H_{19}N_4^+Cl^-HCl$
Activity:	Competes with thiamine active transport preventing coccidian merozoite production
Clopidol	
Synonym:	3,5-dichloro-2,6-dimethyl-4-pyridinol
Chemical formula:	$C_7H_7C_{l2}NO$
Activity:	Prevents coccidian oocyst shedding
Decoquinate	
Synonym:	Ethyl-6-(decycloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylate
Activity:	Kills coccidia by interfering with cellular respiration
Monensin	
Synonym:	2-[5-ethyltetrahydro-5-[tetrahydro-3-methyl-5-[tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl]-2-furyl-9-hydroxy-beta-methoxy-alpha, gamma, 2, 8-tetramethyl- 1, 6-dioxaspiro[4.5]decane-7-butyric acid
Chemical formula:	$C_{36}H_{61}O_{11}Na$
Activity:	Renders membrane permeable to Na^+ and K^+ ions, allowing excess water into the cell (sporozoite) and affecting mitochondrial activity
Nicarbazin	
Synonym:	Urea N, N′-bis (4 nitrophenyl) compound with 4,6-dimethyl-2 (1H)-pyrimidinone
Chemical formula:	$C_{19}H_{18}N_6O_6$
Activity:	Acts at mitochondrial level, inhibiting energy metabolism
Salinomycin sodium	
Chemical formula:	$C_{42}H_{69}NaO_{11}$
Activity:	Activity similar to that of monensin

tained in a 15°C incubator to the theront stage. To determine the number of theronts within each batch, 500 µl of the theront culture was incubated for 5 min with 200 µl of 0.01% neutral red stain before the addition of 300 µl 10% buffered formalin. The number of viable theronts was determined with a 1 ml Sedgewick-Rafter counting chamber on an Olympus BH2 compound microscope at ×40. Groups of fish were infected together by the addition of ca. 2000 theronts per fish in static water for a minimum of 3 h in the dark (60 to 100 fish of 6.5 g each infected in 15 l). At the end of the theront exposure period, the water flow was restarted and the outflow rates for the tank adjusted; 24 h post-infection, fish were allocated to their respective experimental tanks.

In-feed trials. Once infected, 10 to 15 *Oncorhynchus mykiss* fingerlings were randomly allocated to each 10 l tank and the appropriate chemotherapy regime was started. Trials comprised 3 experimental and 3 control replicates except where insufficient infective stages allowed the use of only 2 replicates. Fish were fed a 1% body weight ration of either a normal pelleted diet top-coated with 20 µl cod liver oil or a medicated diet top-coated with 20 µl cod liver oil for 10 d (Days 3 to 12 post-infection). Amprolium hydrochloride (Sigma), clopidol (Coyden 25, Rhone Merieux), decoquinate (Deccox, Rhone Merieux), monensin (Sigma), nicarbazin (Sigma) and salinomycin sodium (Sacox 120, Hoechst Roussel Vet) were incorporated into a pelleted feed at a nominal concentration of 100 ppm. The weight of the appropriate drug to be added was subtracted from the daily feed ration so that when the drug was added the total weight of drug and feed in each tank was the same. A 10 d feed supply, for each of the tanks in any given treatment, was made up at a time. Trout pellets and the drug were mixed in a plastic, lidded vessel and rotated for 20 min to ensure even coverage of the trout pellets with drug. At the end of each day, the feed ration for the next day was rotated to ensure even coverage, weighed out into a plastic vial (0.975 g for 15 fish of 6.5 g each) and 20 µl cod liver oil was added. The vials were shaken, ensuring an even coverage of oil, and then left overnight to ensure absorption of the oil binding the drug to the pellets. The following day, the experimental fish were fed 2 rations: 75% in the first hour and the remainder 3 h later. Approximately 1 h after administering the second ration, the uneaten pellets were collected by siphon from the bottom of tanks. Uneaten pellets were dried and weighed and the percentage of food consumed was calculated.

Two treatment regimes were tested. Fish were either fed medicated feed for 10 d prior to infection (Expt 1) or fed medicated feed commencing 1 full day after infection with *Ichthyophthirius multifliis* (Expts 2 to 6). This established whether a compound could provide protection against stages of the parasite at anticipated periods of high infection or, administered immediately, could kill stages of the parasite in fish already infected. Experiments were terminated on Day 13 post-infection when the fish were killed by an overdose of 650 ppm phenoxyethanol, and the total number of *I. multifiliis* trophonts on the excised gills and body surfaces were counted at ×4 magnification using an Olympus SZ40 dissecting microscope.

Statistics. Trophont counts were subjected to normality (Anderson-Darling) and homogeneity of variance tests (*F*-test, Bartlett's, Levene's). If the conditions of normality were satisfied then parametric tests were applied (2-sample *t*-test, ANOVA, Tukey-Kramer multiple comparisons test). If the data were not normally distributed or the variances were heterogeneous, then non-parametric tests (Kruskal-Wallis, Dunn's, STP test, Mann-Whitney) were applied.

RESULTS

Trials in which the fish were given a 10 d treatment of either 100 ppm amprolium hydrochloride (actual ingested dose 103.8 ± 11.9 ppm) or clopidol (actual ingested dose 97.8 ± 12.3 ppm) before exposure to theronts of *Ichthyophthirius multifiliis* significantly reduced the number of trophonts establishing in naïve fish by 62% ($p < 0.0001$%) and 35.2% ($p < 0.01$%) respectively (Expt 1, Table 2). A dose of 100 ppm salinomycin sodium (actual ingested dose 63.4 ± 21.3 ppm) included in the fish feed reduced trophont numbers by 28.8%, but not significantly so in this particular experiment. The trials with decoquinate, monensin and nicarbazin were not effective and were not tested further. Amprolium hydrochloride, clopidol and salinomycin were tested further to determine their efficacy as chemotherapeutants in fish already infected with *I. multifiliis* (Expt 2, Table 2). A dose of 75.2 ppm amprolium hydrochloride administered for 10 d to infected fish brought about trophont reductions of 32.1% (Expt 2, Table 2). A repeat experiment whereby 62.8 ppm amprolium hydrochloride was ingested brought about a 74.7% reduction in trophont numbers (Expt 3, Table 2). A dose of 92.2 ppm clopidol, however, gave only significant results in 1 of the 3 replicate tanks and a 20.1% reduction in trophont numbers across all tanks. Of the 3 compounds tested, salinomycin sodium appeared to be the most efficacious. When infected fish were given a nominal dose of 100 ppm, with an actual ingestion rate of 47.5 ppm, trophont numbers were significantly reduced by 93.4% (Expt 2, Table 2). Repeat experiments feeding fish a dose of 100 ppm (actual ingestion rates of 37.8

Table 2. *Oncorhynchus mykiss*. Efficacy of each in-feed compound as a treatment for *Ichthyophthirius multifiliis* infection in flow-through tank trials (except where stated otherwise). Regime, number of trophonts (mean ± SD) for control and treated fish (number of fish), and statistical significance of results. ns: not significant; sig: significant (level of significance); T1–3: test tanks; C1–3: control tanks

Compound/regime	Nominal dose (ppm)	No. of trophonts fish^{-1} (n) Test	Control	Significance
Expt 1: *Feed (100 ppm) administered for 10 d prior to parasite exposure (static tank trials)*				
Amprolium hydrochloride	103.81 ± 11.92	38.36 ± 27.49 (14)	101.05 ± 43.08 (19)	Sig. ($p < 0.0001$)
Clopidol	97.82 ± 12.27	65.44 ± 37.93 (16)		Sig. ($p < 0.01$)
Decoquinate	101.34 ± 18.43	97.90 ± 36.26 (14)		ns
Monensin	72.57 ± 25.72	110.00 ± 42.94 (15)		ns
Nicarbazin	96.08 ± 46.22	142.67 ± 56.34 (6)		ns
Salinomycin sodium	63.41 ± 21.30	71.90 ± 29.91 (10)		ns
Expt 2: *Feed (100 ppm) administered for 10 d after parasite exposure*				
Amprolium hydrochloride	70.20 ± 20.86	19.40 ± 7.31 (10)	56.13 ± 29.35 (8)	Sig. (T1 vs C2 $p < 0.05$, T1 vs C3 $p < 0.001$)
	72.41 ± 21.78	35.13 ± 18.29 (8)	79.50 ± 24.64 (10)	
	82.91 ± 19.83	152.00 ± 69.07 (8)	168.30 ± 48.09 (10)	
	avg. = 75.17 ± 20.82			
Clopidol	95.54 ± 33.57	29.63 ± 26.15 (8)	56.13 ± 29.35 (8)	Sig. (T1 vs C3 $p < 0.01$)
	83.14 ± 28.08	57.40 ± 18.53 (10)	79.50 ± 24.64 (10)	
	98.03 ± 31.67	155.78 ± 66.01 (8)	168.30 ± 48.09 (10)	
	avg. = 92.24 ± 31.11			
Salinomycin sodium	48.05 ± 9.21	1.25 ± 1.39 (8)	56.13 ± 29.35 (8)	Sig. (T1 vs C1 $p < 0.01$, T1 vs C2 $p < 0.001$, T1 vs C3 $p < 0.001$, T2 vs C1 $p < 0.05$, T2 vs C2 $p < 0.01$, T2 vs C3 $p < 0.001$, T3 vs C2 $p < 0.001$, T3 vs C3 $p < 0.001$)
	55.52 ± 6.35	5.86 ± 3.31 (8)	79.50 ± 24.64 (10)	
	38.88 ± 25.37	13.00 ± 10.38 (9)	168.30 ± 48.09 (10)	
	avg. = 47.48 ± 13.64 (100)			
Expt 3: *Dose and exposure as Expt 2*				
Amprolium hydrochloride	58.20 ± 19.69	30.11 ± 19.34 (9)	105.40 ± 63.07 (15)	Sig. (T1 vs C1 $p < 0.001$, T1 vs C2 0. $p < 0.001$, T2 vs C1 $p < 0.001$, T2 vs C2 $p < 0.001$)
	67.40 ± 20.27	22.67 ± 9.21 (9)	103.54 ± 59.34 (13)	
	avg. = 62.80 ± 19.7			
Expt 4: *Dose and exposure as Expt 2*				
Salinomycin sodium	39.50 ± 9.97	4.40 ± 3.30 (10)	46.20 ± 24.60 (10)	Sig. (T1 vs C1 $p < 0.01$, T1 vs C2 $p < 0.01$, T2 vs C2 $p < 0.05$)
	36.30 ± 13.23	15.80 ± 9.30 (10)	55.70 ± 18.20 (10)	
	avg. = 37.80 ± 11.60 (100)			
Expt 5: *As Expt 2 but feed administered at dose of 50 or 100 ppm*				
Salinomycin sodium (50 ppm)	24.55 ± 6.38	33.70 ± 24.40 (10)	43.40 ± 41.50 (10)	ns
	24.65 ± 6.59	46.90 ± 38.40 (10)	53.20 ± 26.00 (10)	
	avg. = 24.60 ± 6.50 (50)			
Salinomycin sodium (100 ppm)	41.71 ± 8.04	21.70 ± 16.50 (10)	43.40 ± 41.50 (10)	Sig. (T1 vs C2 $p < 0.01$, T2 vs C2 $p < 0.001$)
	43.89 ± 7.43	5.40 ± 4.40 (10)	53.20 ± 26.00 (10)	
	avg. = 42.80 ± 8.10 (100)			
Expt 6: *As Expt 2 but feed administered for 5 d after parasite exposure*				
Salinomycin sodium	46.40 ± 13.37	30.00 ± 29.00 (10)	37.00 ± 18.20 (10)	ns
	40.60 ± 5.56	69.40 ± 62.00 (10)	12.10 ± 18.60 (10)	
	avg. = 43.10 ± 9.50 (100)			

and 42.8 ppm) also significantly reduced trophont numbers by 80.2 and 72% respectively (Expts 4 and 5, Table 2). As the experimental fingerlings within this trial showed palatability problems with salinomycin sodium, a lower nominal dose of 50 ppm was tested (Expt 5, Table 2). The palatability problems remained, and the actual dose taken up by the fish was only 24.6 ppm; there was no significant difference in the number of trophonts between those receiving medicated feed and the control group. A further trial was carried out in which medicated feed was fed for only 5 d at a nominal dose of 100 ppm (Expt 6, Table 2). The

actual ingestion rate was 43.1 ppm, but there was no significant difference between the test and control groups. These results indicate that salinomycin sodium is an effective treatment for *I. multifiliis* when given at the nominal dose of 100 ppm for 10 d.

DISCUSSION

Of the 6 in-feed compounds tested, the trials conducted with amprolium hydrochloride and clopidol (Expts 1 to 3) showed some protection against subsequent exposure to theronts of *Ichthyophthirius multifiliis* (Table 2). The period of protection provided by these compounds is unknown, and is worthy of further investigation. If the protection is long-lasting then these compounds could have their place in a management control strategy whereby they could be administered to farm stock prior to periods of high infection or prolonged elevation of water temperature. It is more often the case, however, that treatments are not given until the white spots or trophonts are detected on the fish. Thus it is imperative to have efficacious chemicals that are able to kill the trophonts intra-epithelially. In-feed treatments with amprolium hydrochloride or clopidol for 10 d in fish already infected with *I. multifiliis* had significant results, reducing trophont numbers by 32.1 to 74.7 and 20.1% respectively. The best results, however, were obtained with salinomycin sodium, with doses ranging from 37.8 to 47.5 ppm reducing trophont numbers by 72 to 93.4%. The target inclusion rate of 100 ppm for 10 d appears to be quite critical for this compound, as lower target doses of medicated feed (nominal dose of 50 ppm = 24.6 ± 6.5 ppm actual ingested dose) or shorter periods of administration (5 d) resulted in a lower removal of trophonts. Despite the efficacy of salinomycin sodium as an anti-coccidial substance that causes parasite death through mitochondrial and metabolic disturbance as a consequence of increased membrane permeability and osmotic disruption, poultry receiving medicated feed (doses of 60 to 180 ppm) were reported to have lower mean body weights than control groups (Rizvi & Anjum 1999), suggesting unpalatability of this drug.

Data relating to the retention of these drugs within body tissues derive primarily from studies conducted within the poultry industry. Atef et al. (1993) administered a single dose of salinomycin orally to chickens (20 mg kg^{-1} body weight) and found salinomycin to have a half-life of 3.64 h and an elimination half-life of 1.96 h. After 48 h, no salinomycin residues were detected in tissues except in the liver, but these had disappeared completely 72 h after administration of the drug. In a similar study by Hamamoto et al. (2000), chickens in both a fasted and a non-fasted condition were fed a diet containing either 13 or 26 mg kg^{-1} amprolium hydrochloride. Amprolium residues in plasma samples taken from the sub-wing vein indicated that the elimination half-life after oral administration was 0.292 to 0.654 h. Oishi (1991) fed chickens a medicated feed containing 1 ppm clopidol for 10 d. Clopidol at an average level of 0.036 ppm was detected in the eggs throughout the course of treatment, and was no longer detectable 3 d after the chickens returned to an unmedicated diet. Given that the activity of these drugs is the same in trout, to maximise exposure of the parasite to the drug, fish are therefore best treated after infection with *Ichthyophthirius multifiliis*. A course of medicated feed prior to periods of high parasite exposure would therefore confer little protection, unless continued, since the drugs are rapidly metabolised and eliminated from the body.

The number of potentially efficacious compounds for *Ichthyophthirius multifiliis* is possibly being underestimated because either the screening methodology used by some studies is inappropriate or because a chemical is rejected as it failed to eradicate all stages of the parasite. Considering the relative ineffectiveness of the chemicals presently permitted for the treatment of *I. multifiliis*, chemicals that achieve significant reductions in the number of surviving trophonts are certainly of value. Although, under the regimes tested here, salinomycin sodium does not completely eradicate infection, it is still of great potential value as a treatment within a programme of management control. If therapy leads to the survival of fish exposed to a first infection of *I. multifiliis*, then an acquired immunity could provide subsequent protection (up to 8 mo, Hines & Spira 1974) against further infection in an environment of continual exposure.

A study by Tojo & Santamarina (1999), screening potential chemotherapeutants for use against *Ichthyobodo necator*, demonstrated the efficacy of 3 promising candidates, metronidazole, secnidazole, triclabendazole. Subsequently, Tojo-Rodriguez & Santamarina-Fernandez (2001) tested metronidazole and secnidazole against *Ichthyophthirius multifiliis*, whereby efficacy was assessed by trophont counts in skin scrapes. Because no one chemical completely eradicated trophonts, these authors concluded that neither of these compounds was efficacious. A closer look at the latter data set shows that 35% of the fish (20 per test group) tested with metronidazole and 75% of the fish tested with secnidazole were free of trophonts compared to 5% of fish in the control group. Their methodology makes it difficult to assess the true value of these compounds, and they should therefore be retested taking trophont counts from the whole body and gills.

This study has demonstrated the efficacy of a 10 d course of medicated feed containing salinomycin in

reducing the number of surviving trophonts on experimentally infected fish by 72 to 93.4%. The protection offered to fish by this compound in their feed remains to be established. It would be worthwhile to determine if the results could be improved upon by using salinomycin in combination with other anti-coccidiostats in a shuttle program, as has been successfully done in the poultry industry.

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