ブリ類のべこ病治療薬の探索

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Research article

Chemical Treatment of Beko Disease in *Seriola* Fishes: Laboratory Trials of Commercially Available Fishery and Veterinary Medicines

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ABSTRACT—Beko disease, caused by infection of the microsporidian *Microsporidium seriolae*, results in significant damage to farmed amberjack, *Seriola* spp., in Japan. Little is known about the disease, including the general biology of the causative agent, and no therapeutic method has been established to treat it. We determine the therapeutic efficacies of various drugs by administering them in-feed to juvenile *Seriola* spp. naturally infected with *M. seriolae*. Candidate drugs include commercially available fishery anthelminthic, febantel, and eight other compounds. Fish were given various doses of drugs, and *M. seriolae* infection in their trunk muscle was then checked by quantitative PCR (qPCR) and macroscopic cyst detection. Fish treated with febantel at an early stage of infection had significantly lower rates of cyst formation and qPCR-positive. However, febantel administered to fish after cyst formation did not reduce cyst numbers. Other drugs showed no apparent efficacy to prevent cyst formation or to promote cyst reduction. Results indicate febantel is a promising therapeutic agent for beko disease when applied at an early stage of infection.

Key words: Febantel, Fenbendazole, Microsporidia, *Seriola dumerili, Seriola quinqueradiata*

Beko disease in commercially farmed amberjacks, *Seriola* spp., has been known in Japan for many years (Egusa, 1982). The disease is caused by infection of the microsporidian *Microsporidium seriolae,* and forms unsightly whitish cysts to 1 cm length in fish somatic muscle. Within the cytoplasm of muscle cells, the parasite replicates and forms spores, so cysts may contain all of meronts, sporonts, sporoblasts, and spores (Egusa, 1982). As the infection matures the cysts disintegrate, resulting in characteristic concave depressions (beko) on the fish body surface.

Historically, beko disease primarily afflicted juvenile *Seriola quinqueradiata* reared in restricted geographical areas. Also, most affected fish recovered by the time they reached harvest size (Sano *et al.*, 1998; Yokoyama *et al.*, 1996). Accordingly, *M. seriolae* was considered to have relatively low pathological effects on fish, and the unpleasant cysts found on a small proportion of harvested fish in a limited number of fish farms were not of great concern. However, since around 2012, outbreaks of beko disease have occurred in various localities

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where intensive *Seriola* farming occurs, such as southeast of Shikoku and southern Kyushu areas. In these recent outbreaks, fish with severe infections have reduced growth, and disease-related mortality has become evident. Moreover, beko diseases have been reported for *Seriola dumerili* and *Seriola lalandi* also. Heavily infected fish retain cysts at harvest age, resulting in reduced market value and significant economic losses to fish farmers (Yokoyama, 2017).

Similar microsporidian diseases (beko and/or cyst formations in somatic muscle) have been reported for other farmed marine fish, such as *Pagrus major*, *Verasper variegatus,* and *Thunnus orientalis*; causative agents have been tentatively identified as *Microsporidium* sp. RSB, *Microsporidium* sp. SH, and *Microsporidium* sp. PBT, respectively (Egusa *et al.*, 1988; Yokoyama *et al.*, 2008; Zhang *et al.*, 2010). Although spore morphologies of these *Microsporidium* taxa appear to differ, their precise taxonomy is unknown because of highly conservative genetic information, even within different hosts (Mekata *et al.*, 2021). Furthermore, limited information on the general biology of *Microsporidium* taxa is available. Injection or intubation of *M. seriolae* spores from infected *S. quinqueradiata* to naïve conspecifics failed to establish infection, so this parasite most likely utilizes an intermediate invertebrate host to complete its lifecycle (Yokoyama, 2017). However, the lifecycle of no *Microsporidium* species has been determined, and even their infective stage and routes of infection remain unknown. Therefore, experimental infection of *M. seriolae* has not been achieved to date.

Several studies have contributed to the control of beko disease. Sano *et al.* (1998) reported *M. seriolae* infections occurred mainly during May to July, and to peak in June. Yokoyama *et al.* (2011) reported larger *S. quinqueradiata* (> 100 g) to be more tolerant to infection than smaller fish. For other fish microsporidia, efficacies of fumagillin were reported for *Heterosporis anguillarum* (= *Pleistophora anguillarum*) in *Anguilla japonica* (Kano *et al.*, 1982) and *Glugea plecoglossi* in *Plecoglossus altivelis* (Takahashi and Egusa, 1976). However, research on treatments for *M. seriolae* infection are limited, with no practical control established. We aim to develop a practical and effective drug-oriented therapeutic treatment against beko disease by testing commercially available veterinary and fishery medicines to determine their efficacy to prevent and/or reduce formation of *M. seriolae* cysts in *Seriola* fish.

Materials and Methods

We conducted seven experiments (Expt. 1–7) using various drugs and administration regimes, at several institutions, at various times. All experimental fish (juvenile *S. dumerili* or *S. quinqueradiata*) were naturally infected with *M. seriolae* either through exposure of the fish in an infested area (a culture site with beko disease outbreaks) for a certain period, or by selecting fish with "beko" symptoms (characteristic concaves and discolorations on the body surface). Experimental and fish conditions are summarized in Table 1. Drugs/chemicals were selected from commercially available fishery and veterinary medicines in accordance with an administration regimen detailed in Table 2. The administration regimes followed those instructed by the manufacturers, except for praziquantel (Experiment 3) and febantel (Experiment 3 and 7) for which various doses were tested. All drugs were administered orally via drug-coated commercial extruded pellets (EP) [various manufacturers and sizes, and coating agents] with the daily feeding rate of 1–2.5% body weight. All experiments were controlled with fish treated in the same general manner, but not given drug feed.

Experimental fish

A total of 7 experiments were performed. Experiment 1 – 3 assessed the efficacy of a total of 9 candidate drugs on the fish at early stages of *M. seriolae* infection. Experiment 4 compared the efficacy of two benzimidazole drugs (febantel and albendazole). Experiment 5 and 6 tested the drug efficacy on the fish with visible *M. seriolae* cysts as well as the effect on spore viability. Experiment 7 was conducted to determine the minimum effective dose of febantel. The infection status of the experimental fish for each experiment was as follows.

Experiment 1 tested four drugs/chemicals on juvenile *S. dumerili* that had been immersed in infested water for 17 days. The gene level of *M. seriolae* in seawater during the exposure period ranged from 3.39×10^0 to 6.29×10^2 copies/L. While some fish tested PCR-positive for *M. seriolae*, cysts were not detected in somatic muscles; they were considered to be at an early stage of infection (pre-cyst formation). Experiments 2 and 3 tested five drugs on *S. dumerili* immersed in infested water for 12 days. The gene level of *M. seriolae* in seawater during the exposure period ranged from 2.01×10^2 to 5.74×10^2 copies/L. In contrast to experiment 1, a small percentage of fish (9.7%) in experiments 2 and 3 harbored visible cysts in the muscle; they were considered to be at a "late-early stage" (cyst-forming stage) of infection. Experiments 4–6 used fish with high cyst detection rates (Table 1), representing typical beko-diseased fish that were considered to be at a mid- or post-cyst formation stage. Experiment 7 was conducted to determine the minimum effective dose of a drug shown to be effective in previous experiments (Table 3). The *S. quinqueradiata* used in experiment 7 were immersed in infested water for 17 days, after which none had visible cysts (pre-cyst formation stage).

Table 1. Summary of experiments 1–6. Body weight (BW) of sampled fish, cyst formation rate (cyst detection rate), mean number of cysts*, and gene detection rate with average copy number** before and after administration of candidate drugs, as well as spore survival rate (determined by acridine orange staining)

	Drug: Dosage (mg/kg/day)	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg
Expt. 1		Pre-drug administration					11 d post-last-drug administration					36 d post-last-drug administration				
Seriola dumerili	Non-drug control	17.0 ± 2.9	0% (0/30)	NA	6.7% (2/30)	NA	18.6 ± 5.4	0% (0/10)	0	NA	NA	22.8 ± 5.5	52.4% (22/42)	11.6 ± 6.2 (4–25)	83.3% (35/42)	3.90×10^{7} $\pm6.60\times10^{7}$
2016.7.1–8.3	Lysozyme hydrochloride: 10.0	〃	〃	〃	〃	〃	20.6 ± 4.4	30% (3/10)	4.7 ± 1.2 (4–6)	NA	NA	23.9 ± 7.3	66.7% (24/36)	12.6 ± 8.4 (1–34)	86.1% (31/36)	8.09×10^{7} $\pm9.27\times10^{7}$
21.8–23.1°C	Mixture of sulfamonomethoxine hydrate & Ormetoprim mix: 12.5	〃	〃	〃	〃	〃	19.5 ± 3.6	10% (1/10)	8.0	NA	NA	23.0 ± 5.0	61.3% (20/33)	13.9 ± 6.9 (4–24)	93.9% (31/33)	6.52×10^{7} $\pm6.64\times10^{7}$
	Amprolium hydrogen chloride: 40.0	〃	〃	〃	〃	〃	18.8 ± 4.4	10% (1/10)	13.0	NA	NA	22.6 ± 6.4	59.5% (22/37)	9.7 ± 6.1 (2–24)	97.3% (36/37)	5.56×10^{7} $\pm9.55\times10^{7}$
	Monensin sodium salt: 40.0	〃	〃	〃	〃	〃	17.8 ± 3.1	10% (1/10)	3.0	NA	NA	23.9 ± 10.3	55.0% (22/40)	11.2 ± 7.1 (2–30)	90.0% (36/40)	4.25×10^{7} $\pm8.76\times10^{7}$
Expt. 2		Pre-drug administration					11 d post-last-drug administration					35 d post-last-drug administration				
Seriola dumerili	Non-drug control	38.0 ± 7.9	9.7% (3/31)	NA	NA	NA	46.0 ± 6.0	20.0% (2/10)	5.0 ± 5.7 (1–9)	NA	NA	49.2 ± 7.3	55.6% (20/36)	6.8 ± 6.7 (1–26)	70.0% (14/20)	1.89×10^{7} $\pm4.60\times10^{7}$
2016.7.12–8.18	Sulfamoyldapsone: 2.5	〃	〃	〃	〃	〃	46.4 ± 2.7	40.0% (4/10)	8.8 ± 1.5 (7–10)	NA	NA	45.0 ± 11.3	61.5% (24/39)	6.2 ± 5.2 (1–20)	80.0% (16/20)	1.22×10^{8} $\pm2.20\times10^{8}$
22.2–25.8°C	Teflubenzuron: 5.0	〃	〃	〃	〃	〃	41.9 ± 7.3	30.0% (3/10)	5.3 ± 3.8 (1–8)	NA	NA	46.6 ± 5.9	50.0% (20/40)	7.6 ± 5.3 (1–20)	55.0% (11/20)	3.35×10^{7} $\pm8.00\times10^{7}$
	Toltrazuril: 10.0	〃	〃	〃	〃	〃	43.9 ± 6.0	20.0% (2/10)	22.5 ± 3.5 (20–25)	NA	NA	45.3 ± 9.0	57.5% (23/40)	6.1 ± 5.3 (1–24)	75.0% (15/20)	1.84×10^{8} $\pm3.77\times10^{8}$
Expt. 3		Pre-drug administration					11 d post-last-drug administration					37 d post-last-drug administration				
Seriola dumerili	Non-drug control	38.0 ± 7.9	9.7% (3/31)	NA	NA	NA	43.5 ± 7.8	60% (6/10)	6.2 ± 6.0 (1–15)	100% (10/10)	6.53×10^{4} $\pm1.42\times10^{5}$	44.5 ± 5.5	53.8%[a] (21/39)	9.5 ± 5.3 (2–19)	100%[a] (39/39)	4.58×10^{4} $\pm8.31\times10^{4a}$
2016.7.14–8.17	Febantel: 3.1	〃	〃	〃	〃	〃	44.6 ± 7.8	20% (2/10)	8.5 ± 0.7 (8–9)	70% (7/10)	1.42×10^{2} $\pm3.15\times10^{2}$	46.0 ± 5.7	10.0%[b] (4/40)	7.0 ± 5.7 (1–14)	47.5%[b] (19/40)	2.13×10^{3} $\pm7.52\times10^{3b}$
24.5–28.9°C	Praziquantel: 37.5	〃	〃	〃	〃	〃	46.7 ± 4.8	40% (4/10)	10.3 ± 3.4 (7–15)	90% (9/10)	1.76×10^{5} $\pm4.17\times10^{6}$	44.3 ± 6.8	52.5%[a] (21/40)	6.5 ± 4.8 (1–17)	92.5%[a] (37/40)	5.94×10^{4} $\pm1.16\times10^{5a}$
Expt. 4		Pre-drug administration					14 d post-last-drug administration									
Seriola dumerili	Non-drug control	21.4 ± 5.7	40.0% (8/20)	4.25 ± 1.75 (1–6)	75.0% (15/20)	6.92×10^{6} $\pm1.67\times10^{7}$	26.6 ± 7.4	70%[a] (14/20)	5.1 ± 2.7 (2–11)	80.0% (16/20)	3.57×10^{7} $\pm6.32\times10^{7}$					
2016.8.9–8.23	Febantel: 12.5	〃	〃	〃	〃	〃	24.6 ± 5.1	35.0%[a] (7/20)	6.9 ± 2.6 (1–10)	75.0% (15/20)	7.90×10^{6} $\pm1.48\times10^{7}$					
23.8–25.2°C	Albendazole: 15	〃	〃	〃	〃	〃	23.1 ± 5.7	43.5%[a] (10/23)	7.2 ± 4.2 (2–15)	87.0% (20/23)	2.03×10^{7} $\pm2.34\times10^{7}$					
	Febantel: 25	〃	〃	〃	〃	〃	25.8 ± 8.2	28.6%[b] (6/21)	7.3 ± 3.6 (1–12)	66.7% (14/21)	8.52×10^{6} $\pm1.17\times10^{7}$					
	Albendazole: 30	〃	〃	〃	〃	〃	27.7 ± 7.1	47.6%[a] (10/21)	4.8 ± 2.6 (2–11)	66.7% (14/21)	3.03×10^{7} $\pm9.98\times10^{7}$					
Expt. 5		Pre-drug administration					36 d post-last-drug administration					Spore mortality				
Seriola dumerili	Non-drug control	120.6 –132.6	63.6% (35/55)	NA	NA	NA	120.6 ± 16.1	64.7% (11/17)	NA	NA	NA	16.1%[a] (482/2,998)				
2016.8.26–9.30	Albendazole: 15	〃	〃	〃	〃	〃	131.4 ± 17.4	65% (13/20)	NA	NA	NA	52.3%[b] (2,243/4,287)				
23.8–26.6°C	Febantel: 12.5	〃	〃	〃	〃	〃	132.6 ± 15.8	70% (14/20)	NA	NA	NA	37.2%[b] (1,931/5,173)				
Expt. 6		Pre-drug administration					87 (PZQ) or 55 (FBT) d post-last-drug administration					†Mean spore mortality				
Seriola quinqueradiata	Non-drug control	93.1 ± 18.5	100% (11/11)	3.0 ± 2.2 (1–6)	NA	NA	824.0 ± 130.9	91.3% (21/23)	4.2 ± 3.7	NA	NA	13.8 ± 10.8%				
2016.07.02–12.02	Febantel: 25	〃	〃	〃	〃	〃	824.2 ± 125.5	100% (20/20)	4.5 ± 3.1	NA	NA	12.6 ± 8.3%				
16.5–30.7°C	Praziquantel: 150	〃	〃	〃	〃	〃	839.9 ± 131.3	90.9% (20/22)	5.9 ± 3.3	NA	NA	11.1 ± 5.5%				

* Average number of cysts per half body of cyst-harboring fish (visual count).
** Calculated from the mean value of multiple samples in individual fish.
Different letters indicate statistically significant differences (chi-square test, $p < 0.05$)
NA, Data not available.
† Mean value calculated from 20–23 cysts in each treatment group.

Table 2. List of drugs/chemicals used in experiments, their dosage, and administration regime ((administration + cessation) × total cycles)

Experiment	Active pharmaceutical ingredient	Drug name	Manufacturer	Dosage	Regime
1	Lysozyme hydrochloride	Potozyme	ASKA Animal Health Co., Ltd.	10.0 mg/kg/d	(7 d + 3 d) × 3 cycles
	Sulfamonomethoxine hydrate & Ormetoprim mix	Ektecin	Meiji Seika Pharma Co., Ltd.	12.5 mg/kg/d	(5 d + 4 d) × 4 cycles
	Amprolium hydrogen chloride		LKT Laboratories, Inc.	40.0 mg/kg/d	(5 d + 4 d) × 4 cycles
	Monensin sodium salt		Wako Pure Chemical Industries, Ltd.	40.0 mg/kg/d	(5 d + 4 d) × 4 cycles
2	Sulfamoyldapsone	Freetomin powder50	DS Pharam Animal Helth Co., Ltd.	2.5 mg/kg/d	(5 d + 4 d) × 4 cycles
	Teflubenzuron		Wako Pure Chemical Industries, Ltd.	5.0 mg/kg/d	(5 d + 4 d) × 4 cycles
	Toltrazuril		Wako Pure Chemical Industries, Ltd.	10.0 mg/kg/d	(5 d + 4 d) × 4 cycles
3	Febantel	Marinebantel	Meiji Seika Pharma Co., Ltd.	3.1 mg/kg/d	(5 d + 4 d) × 4 cycles
	Praziquantel	Benesaru	ASKA Animal Health Co., Ltd.	37.5 mg/kg/d	(3 d + 4 d) × 5 cycles
4	Albendazole		LKT Laboratories, Inc.	15.0 mg/kg/d	5 d
	〃		〃	30.0 mg/kg/d	5 d
	Febantel	Marinebantel	Meiji Seika Pharma Co., Ltd.	12.5 mg/kg/d	5 d
	〃	〃	〃	25.0 mg/kg/d	5 d
5	Albendazole		LKT Laboratories, Inc.	15.0 mg/kg/d	35 d
	Febantel	Marinebantel	Meiji Seika Pharma Co., Ltd.	12.5 mg/kg/d	(3 d + 2 d) × 5 cycles
6	Febantel	Marinebantel	Meiji Seika Pharma Co., Ltd.	25.0 mg/kg/d	(5 d + 3 d) × 6 cycles
	Praziquantel	Benesaru	ASKA Animal Health Co., Ltd.	150.0 mg/kg/d	(3 d + 7 d) × 6 cycles
7	Febantel	Marinebantel	Meiji Seika Pharma Co., Ltd.	0.5 mg/kg/d	3 d
	〃	〃	〃	〃	5 d
	〃	〃	〃	1.0 mg/kg/d	3 d
	〃	〃	〃	〃	5 d
	〃	〃	〃	3.0 mg/kg/d	3 d
	〃	〃	〃	〃	5 d
	〃	〃	〃	〃	10 d

Detection of M. seriolae *and assessment of drug efficacy*

The efficacy of each drug/chemical was judged by comparing the proportion of PCR-positive and/or cyst bearing individuals, as well as gene copy number, with values for control groups that had been treated in the same general manner but given non-drug feed. Sampling was conducted prior to experimentation, and at various times after drug administration (Tables 1, 3). Cyst detection and tissue sampling for qPCR followed basic protocols; sampled fish were filleted into three pieces and both sides of trunk muscle were sliced at 5 to 10 mm thick. Visible cysts in somatic muscle tissues were then counted to determine mean cyst intensity (i.e. number of cyst in the toral number of cyst bearing fish). A sample of cysts were observed under a microscope to confirm microsporidian spores, for checking spore morphology and/or staining them with Uvitex-2B (Yokoyama *et al.*, 1996). An entire half fillet was used for qPCR; it was placed into a plastic bag and homogenized using a rubber hammer, or other similar tools, with up to 25 mg of homogenized tissue then sampled. DNA extraction used a QIAamp DNA Mini Kit (Qiagen), with qPCR performed following the method developed by Mekata *et al.* (2021). One sample was used for each tested fish and absolute quantification was achieved by using a standard curve, constructed by amplifying known amounts of target DNA in a parallel set of reactions. Samples with apparent amplification curves were considered to be positive for *M. seriolae*.

Assessing spore viability

Viability of spores collected from drug-treated and control fish was assessed and compared in experiments 5 and 6 following the general protocol described in Iida *et al.* (1993). Several cysts were randomly sampled from muscle tissue and homogenized in phosphate-buffered saline (PBS). Spore solution was then centrifuged at 1,700 × *g* for 3 min, and the supernatant was discarded. Acridine orange was added to achieve a final concentration of 0.05 mg/mL; the solution was left for 5 min before observation under a fluorescent microscope. The use of acridine orange for the viability assay of microsporidian spores have been reported previously (Peterson *et al.*, 1988; El-Taweel *et al.*, 2012). Stained spores (brightly fluorescent) in red were considered dead, and the proportion of dead/live spores was calculated for a minimum of 500 spores per sample. Staining and observation were performed within 30 min and 7 h of cyst collection, respectively.

Statistical analysis

Comparison of cyst formation rate, qPCR positive rate, and the proportion of dead/live spores between treatment groups was achieved by chi-square analysis. A Dunnett's test was used to compare estimated gene copy number between groups. An alpha value was set at 0.05 for all analyses.

Table 3. Experiment 7 summary: body weight (BW) of sampled fish, cyst formation rate (cyst detection rate), mean number of cysts*, and gene detection rate with average copy number** before and after administration of different doses of febantel for different durations

	Dose/day	Duration	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg	BW (g)	Cyst detection rate (count)	Mean number of cysts (range)	PCR positive rate (count)	Mean copies/mg
Expt. 7			Pre drug administration					12 d post drug administration (27 d after exposure to infested sea)					35 d post drug administration (50 d after exposure to infested sea)				
Seriola quinqueradiata	Non-drug control							67.6 ± 9.82	30% (3/10)	5.67 ± 4.04 (2–10)	50%[a] (5/10)	1.99×10^{6} $\pm4.16\times10^{6}$	79.3 ± 10.75	68%[a] (17/25)	7.29 ± 4.90 (1–18)	84%[a] (21/25)	4.54×10^{7} $\pm1.29\times10^{8a}$
2018.11.1–12.5	Febantel 0.5 mg/kg	3 d	17.0 ± 2.9	0% (0/30)	NA	6.7% (2/30)	NA	60.5 ± 10.42	40% (4/10)	2.00 ± 0.82 (1–3)	60%[a] (6/10)	4.62×10^{6} $\pm7.71\times10^{6}$	78.8 ± 9.62	65.4%[a] (17/26)	9.13 ± 7.46 (2–29)	88.5%[a] (23/26)	1.08×10^{8} $\pm2.65\times10^{8a}$
18.0–20.2°C	〃	5 d	〃	〃	〃	〃	〃	62.2 ± 8.10	60% (6/10)	3.50 ± 3.08 (1–9)	60%[a] (6/10)	1.18×10^{6} $\pm3.06\times10^{6}$	67.4 ± 9.08	76%[a] (19/25)	8.47 ± 5.23 (2–19)	96%[a] (24/25)	2.64×10^{8} $\pm4.45\times10^{8a}$
	Febantel 1.0 mg/kg	3 d	〃	〃	〃	〃	〃	61.2 ± 5.67	20% (2/10)	9.00 ± 1.41 (8, 10)	50%[a] (5/10)	1.73×10^{6} $\pm3.57\times10^{6}$	75.5 ± 9.87	68%[a] (17/25)	9.29 ± 6.48 (2–19)	96%[a] (24/25)	2.41×10^{8} $\pm5.57\times10^{8a}$
	〃	5 d	〃	〃	〃	〃	〃	57.4 ± 4.95	40% (4/10)	6.00 ± 4.67 (1–11)	60%[a] (6/10)	4.24×10^{6} $\pm1.22\times10^{7}$	76.4 ± 9.73	80%[a] (20/25)	10.05 ± 6.25 (2–27)	92%[a] (23/25)	1.18×10^{8} $\pm1.61\times10^{8a}$
	Febantel 3.0 mg/kg	3 d	〃	〃	〃	〃	〃	56.8 ± 8.40	0% (0/10)	0.0	0%[b] (0/10)	nd	80.7 ± 12.06	32%[b] (8/25)	4.33 ± 3.08 (1–10)	60%[a] (15/25)	4.93×10^{6} $\pm1.11\times10^{7a}$
	〃	5 d	〃	〃	〃	〃	〃	51.0 ± 6.71	0% (0/10)	0.0	40%[a] (4/10)	4.92×10^{4} $\pm7.55\times10^{4}$	76.4 ± 12.04	19.2%[c] (5/26)	2.80 ± 2.17 (1–6)	23.1%[b] (6/26)	2.27×10^{6} $\pm8.07\times10^{6b}$
	〃	10 d	〃	〃	〃	〃	〃	62.1 ± 12.52	10% (1/10)	1.00	20%[a] (2/10)	3.54×10^{4} $\pm8.09\times10^{4}$	75.1 ± 12.05	0%[c] (0/26)	0.0	3.8 %[b] (1/26)	1.08×10^{3} $\pm5.48\times10^{3b}$

* Average number of cysts per half body of cyst-harboring fish.
** Calculated from the mean value of multiple samples in individual fish.
Different letters indicate statistically significant differences (chi-square test $p < 0.05$)
NA, Data not available.
nd, not detected.

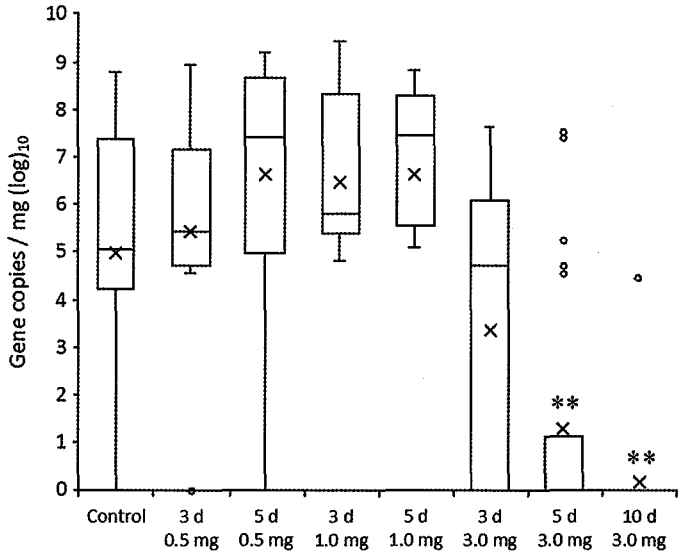

Fig. 1. Log copy numbers of *Microsporidium seriolae* genes in trunk muscle of *Seriola quinqueradiata* at 35 days after receiving different doses of febantel in Expt. 7. Box whisker plots depict the range of gene copies for each group, with the middle line in each box representing the median value, and the lower and upper limits of boxes representing the lower and upper quartiles, respectively. Whiskers extend to lowest and highest copy numbers. Dots and crosses represent outlier and mean values, respectively. Asterisks indicate statistically significant differences from control group samples (**; Dunnett's test, $p < 0.001$).

Results

Drug efficacy

In experiment 1, no drug (lysozyme hydrochloride, a mixture of sulfametonomethoxine hydrate and olometoprim, amprolium hydrogen chloride, or monensin sodium salt) was effective against *M. seriolae*, as cyst detection rate, number of cysts, qPCR positive rate, and gene copy number in treatment groups did not differ from values in control groups 36 days post administration (dpa) (Table 1). Similarly, in experiment 2, *M. seriolae* infection of juvenile *S. dumerili* treated with sulfamoyldapsone, teflubenzuron, or toltrazuril increased at a similar rate to that observed in control fish at 35 dpa.

In experiment 3, the proportion of fish harboring cysts in the control group increased from 9.7% prior to drug administration to 60% and 53.8% at 11 and 37 dpa, respectively (Table 1). In contrast, the proportion of fish harboring cysts in the group given 3.1 mg/kg/d of febantel for 4 weeks (5 days-consecutive administration + 2 days drug cessation period) did not increase, remaining at 10% even at 37 dpa. This rate was significantly lower compared with those of control and praziquantel-treated fish (Table 1, chi-square test, $p < 0.001$). Also, the *M. seriolae* detection rate by qPCR for febantel-treated fish was significantly lower than other groups (chi-square test, $p < 0.001$).

In experiment 4 using fish with a higher initial cyst detection rate (40%), the rate in fishes given different doses of albendazole and febantel remained relatively consistent at 14 dpa (up to 47.6%), whereas that in control fishes increased to 70% (Table 1). At 14 dpa, cyst detection rate in fishes in the 25 mg/kg febantel group was significantly lower than in the control (chi-square test, $p < 0.01$). However, in both experiments 5 and 6 using *S. dumerili* and *S. quinqueradiata* at a late cyst-formation stage with even higher initial cyst detection rates (63.6% and 100%, respectively), all tested doses of febantel, albendazole, and praziquantel, proved ineffective. Cyst detection rates in drug-treated groups remained unchanged even after 1 mo of drug administration (Table 1).

Experiment 7 determined the minimum dose of febantel, which proved to be effective in experiments 3 and 4 on the fish at an early stage of infection. No cysts were observed in fish in this experiment prior to drug administration, although the initial qPCR positive rate was 6.7%. At 12 dpa (18 days from initial sampling), cysts were detected in 30% of control fishes and up to 60% of fish given febantel at doses of 1.0 mg/kg or lower (Table 3). The cyst detection rate in fishes treated daily with febantel at a dose of 3.0 mg/kg did not increase, or did so minimally (to 10%, or 1/10 fishes). The PCR positive rate and gene copy number in fishes in the 3.0 mg/kg group also tended to be lower than in other groups. At 35 dpa there was a further increase in cyst detection rate (up to 80%) in low-dose groups, but rates in the 3.0 mg/kg group were all significantly lower than in control fishes, with a maximum value of 32% (3 days administration, chi-square test, $p < 0.05$). No cysts were detected in fishes treated with 3.0 mg/kg febantel for 10 days, for which the PCR positive rate and gene copy number were significantly lower than those of control fishes (Table 3, Fig. 1). While mean cyst intensity

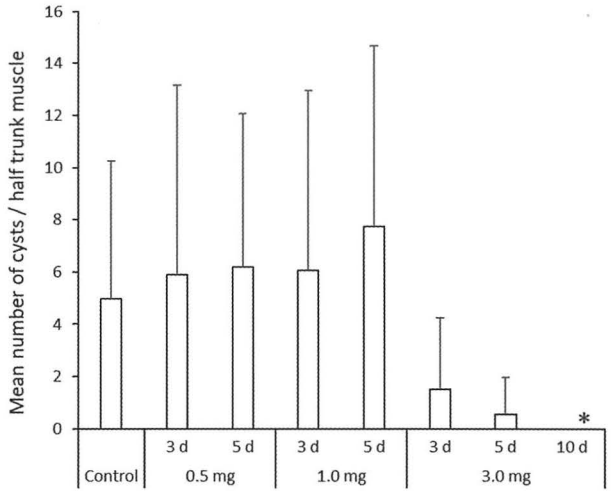

Fig. 2. Mean (+SD) number of *Microsporidium seriolae* cysts in half of the trunk muscle of *Seriola quinqueradiata* given different doses of febantel for 3, 5, or 10 consecutive days. Fish were sampled 35 days post drug administration. An asterisk indicates a statistically significant difference from control fishes (*; Dunnett's test, $p < 0.05$).

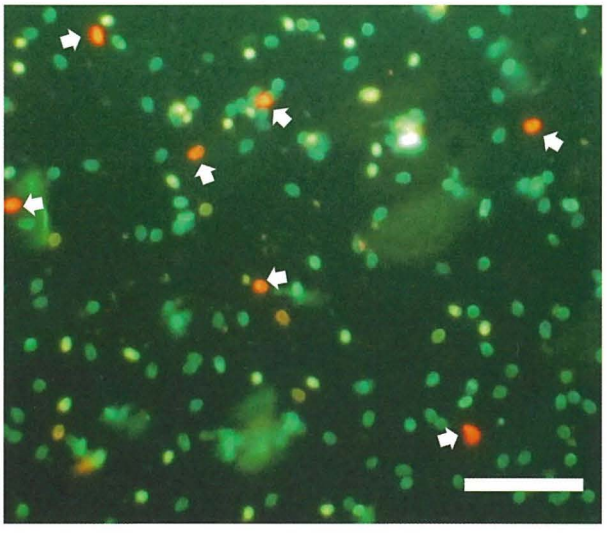

Fig. 3. *Microsporidium seriolae* spores stained with acridine oranges for viability assay in Experiment 5. Brightly fluorescent spores in red (arrows) were considered dead. Scale bar: 20 μm.

at 35 dpa was similar between control, 0.5 mg/kg, and 1.0 mg/kg groups (~ 5–8 cysts/half body), values in the 3.0 mg/kg treatment were considerably lower, and seemed to decline with drug administration duration (Fig. 2).

Spore viability

Results of spore viability assessment varied between experiments. In experiment 5, spore mortality in febantel- (37.2%) and albendazole-treated fish (52.3%) was significantly higher than control fishes (16.1%, chi-square test, $p < 0.01$, Table 1, Fig. 3). In experiment 6, however, spore mortality in febantel- and praziquantel-treated fish was similar to control mortalities (11%–13%, Table 1).

Discussion

We tested the efficacy of 10 drugs/chemical compounds to control *M. seriolae* infection in two species of *Seriola*. Each drug possesses anthelminthic or antibacterial properties, and has been used for veterinary, fishery and/or human medical purposes. The mechanism by which each compound act varies and some exhibit broader spectrum activities against various pathogens than others. Among them, febantel was the only drug that proved to reduce gene copy number and/or prevent *M. seriolae* cyst formation.

Febantel is a prodrug of the fenbendazole (a benzimidazole). Both febantel and fenbendazole are widely regarded to be effective antiparasitics against various nematodes (e.g., *Strongylus* in horses, *Trichuris* and *Ascaris* in pigs), some cestodes (e.g., *Taenia pisiformis* in dogs and cats), as well as off-label or combination use with other drugs against trematodes (e.g., *Alaria* in dogs and cats) and even protozoa (*Giardia* in dogs and cats) (Papich, 2015). Moreover, febantel is approved in Japan for aquaculture usage to treat monogenean gill fluke (*Heterobothrium okamotoi*) infection in tiger puffer *Takifugu rubripes* (Kimura *et al*., 2006). To the best of our knowledge, this is the first report of febantel effectively treating a marine microsporidian, although febantel/fenbendazole is known to effectively treat other microsporidians. For instance, oral administration of fenbendazole (20 mg/kg BW) successfully prevented establishment of *Encephalitozoon cuniculi* in the central nervous system of experimentally challenged rabbits (Suter *et al*., 2001). Fenbendazole and 9 other benzimidazoles showed *in vitro* inhibitory efficacy against replication of the AIDS-associated microsporidian *E. intestinalis* (Katiyar and Edlind, 1997). In fish, fenbendazole and other benzimidazole derivates (albendazole and mebendazole) have been shown to deleteriously affect *Glugea anomala* parasitizing three-spined stickleback *Gasterosteus aculeatus* (Schmahl and Benini, 1998).

The therapeutic effects of benzimidazole compounds occur through binding to beta-tubulin in the target organism and disrupting microtubule-based processes (Robinson *et al*., 2004). Clumped chromatin in the nuclei of meronts, revealed by electron microscopy, indicated the selective anti-tubulin activity of albendazole, with massive disorganization of sporogonic development (Katiyar and Edlind, 1997). The presence of beta-tubulin sequences in *Encephalitozoon* spp. suggests that the benzimidazole sensitivity of microsporidians (Edlind *et al*., 1996), thus the efficacy of fenbenda-

zole against *M. seriolae*, is not entirely surprising.

Although we demonstrated the preventive effects of febantel on multiplication and cyst formation of *M. seriolae,* this drug appeared to be ineffective in eliminating cysts that had already formed in trunk muscle of the host fish. It is also unclear whether the drug has killing effects on *M. seriolae* spores as the results of the two viability assays (Experiment 5 and 6) differed. The reason(s) for such difference is unknown. Staining procedure, judgement of live/dead spores, and other experimental conditions might have influenced the results. *In vitro* assay should also be performed to determine the actual killing effect of the drug. Nevertheless, our results showed that febantel has minimal or no effect on diminish of *M. seriolae* cysts that had already been formed prior to the administration. A similar result was reported in the *E. cuniculi*-rabbit system, in which administration of fenbendazole prior to experimental infection showed some degree of protection, while drug administration 28 days after infection showed no therapeutic effect (Abu-Akkada and Oda, 2016). While the effects of fenbendazole (also albendazole and mebendazole) on *G. anomala* in sticklebacks were most prominent on merogonic and sporogonic stages, drugs at higher doses (fenbendazole; 50 μg/mL for 6 h) also affected mature spores that showed abnormality, damaging cytoplasm membranes and the polaroplast (Schmahl and Benini, 1998). Naturally infected sticklebacks harboring various developmental stages of *G. anomala* were exposed to the drug-containing-water for several hours (Abu-Akkada and Oda, 2016). It is possible that continuous exposure to the drug, rather than oral administration, may have a greater effect on spores. Additionally, doses used in our study might be too low to affect mature spores.

We identified *M. seriolae* infection of *Seriola* fish by qPCR as early as 3–5 days after exposure to infested water. These fish developed cysts in trunk muscle within next 12 days, indicating *M. seriolae* developed relatively fast, with as short as 15 days required to initiate cyst formation. Based on these results, febantel should be administrated before cyst formation, with early infection detection being essential for effective drug treatment. Whilst the qPCR used in our study effectively detected infection at an early stage, more convenient and less time-consuming methods for fish farmers are desirable. Mekata *et al.* (2021) have developed loop-mediated isothermal amplification (LAMP) to facilitate detection of *M. seriolae*. Rapid detection of infection and fast drug administration are probably central to drug-therapy treatment of beko disease.

No tested drug was lethally toxic to juvenile *S. dumerili* or *S. quinqueradiata*. Moreover, no obvious negative effect was apparent on fish growth in any drug-treated group. However, benzimidazoles can be toxic to vertebrates. For instance, nocodazole and parbendazole were highly toxic to monkey kidney cells (Katiyar and Edlind, 1997). Fenbendazole, albendazole and other benzimidazoles are toxic in some birds (Vergneau-Grosset and Larrat, 2016). Pigeons given albendazole and fenbendazole lost weight, and had marked leucopenia and bone marrow hypoplasia (Howard *et al.*, 2002). Albendazole may also cause bone marrow suppression in dogs and cats (Stokol *et al.*, 1997). In fish, signs of toxicity ranging from abnormal swimming to death were observed in rainbow trout exposed to albendazole, oxibendazole, thiabendazole, and fenbendazole (Tojo *et al.*, 1992). While the drug dosage and administration regime that demonstrated efficacy in our study is safe and practical for use in *Seriola* farms, the safety of febantel should be more widely considered. The potential toxicity of febantel on *Seriola* fish was assessed in more detail separately (Shirakashi *et al.*, 2021).

In conclusion, oral administration of febantel at a dose of 3.0 mg/kg BW/day or greater for 3 days is effective at preventing cyst formation and multiplication of *M. seriolae* in trunk muscle of *Seriola* spp., if administered at early stage of infection. However, these results are based on tank experiments under controlled conditions. Further evaluation was conducted separately to determine actual effective doses and administration regimes under farming conditions (Kawakami *et al.*, 2021). Other drugs may be similarly effective against *M. seriolae*, such as fumagillin and other benzimidazoles, which have demonstrated therapeutic efficacy against fish microsporidia (Takahashi and Egusa, 1976; Kano *et al.*, 1982; Schmahl and Benini, 1998). However, because febantel is already approved for aquaculture usage, and is commercially available as a fishery drug in Japan, we believe it has high potential to be a new therapeutic solution for beko disease of *Seriola* spp., and possibly other farmed fishes also.

Acknowledgements

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ブリ類のべこ病治療薬の探索

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ブリ類のべこ病は生活環など不明な点も多く，未だ効果的な治療法はない。そこでべこ病の治療法を確立するため，*Microsporidium seriolae* に感染したブリ類稚魚に対し，フェバンテル他 8 薬剤を用いた投薬試験を行い，体側筋中のシストの有無と原因虫の遺伝子検出により治療効果の有効性を評価した。その結果，感染初期にフェバンテルを投与した魚で，シスト形成率および遺伝子検出率が有意に低く，本薬がべこ病原因虫のシスト形成や体側筋中での増殖を抑制することが示された。一方で，既にシストが高い割合で形成された罹患魚群の本薬投与試験では，顕著な治療効果は認められなかった。以上から，シストが形成される前の感染初期に本薬を経口投与すればシストの形成を抑えられることが明らかとなった。

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